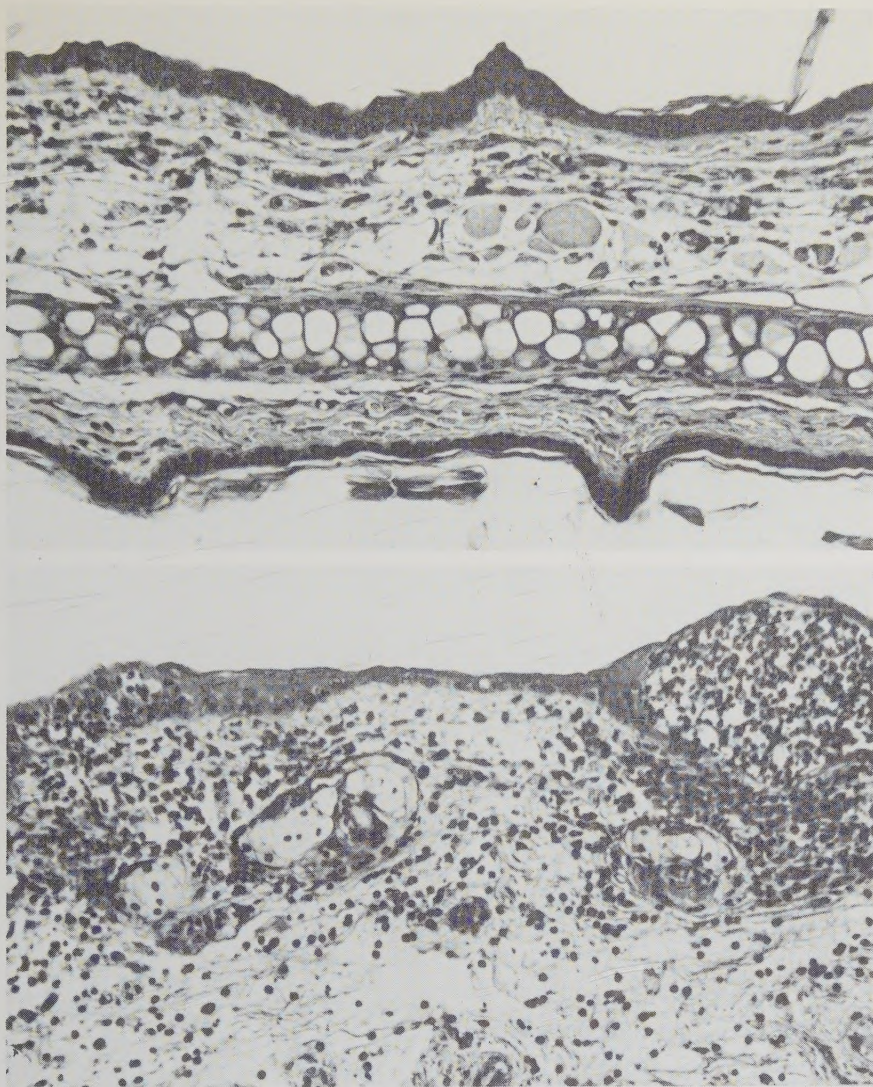




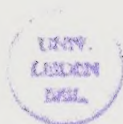
Immunological Effects of Diethylstilbestrol and Estramustine Phosphate.

By
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1984

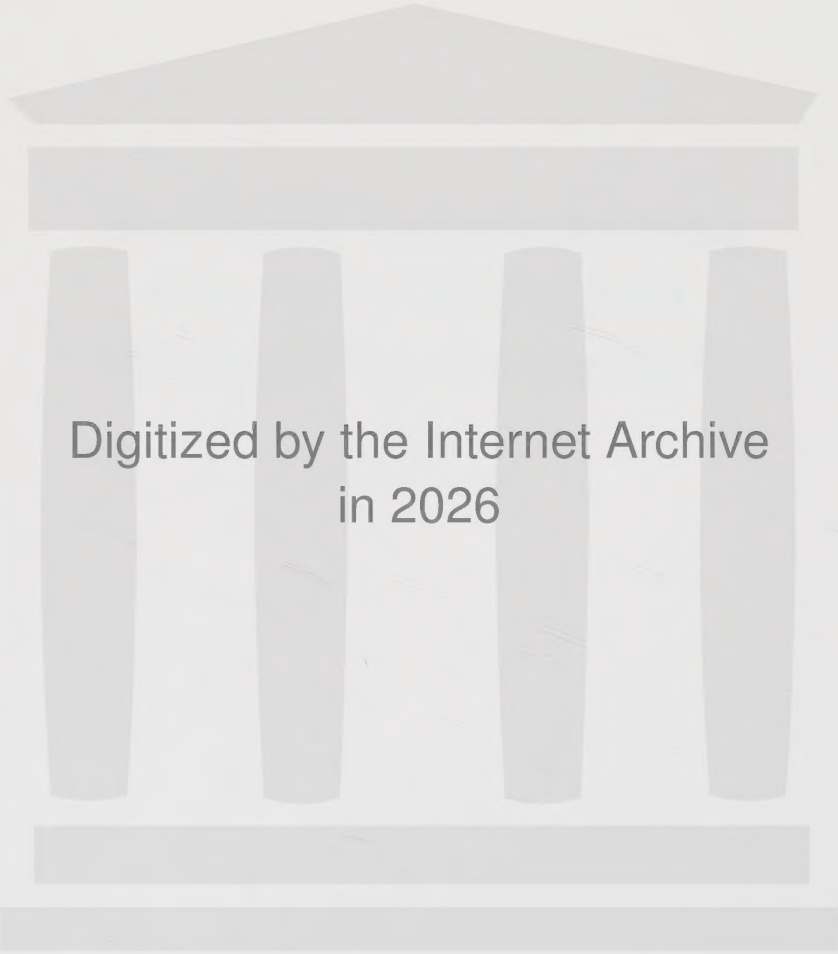


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Immunological Effects of Diethylstilbestrol and Estramustine Phosphate

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To my family

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This thesis is based on the following papers, which will be referred to by their Roman numerals:

- I Effect of Diethylstilbestrol and Estramustine phosphate (Estracyt®) on delayed hypersensitivity response to oxazolone in male mice.
Svein A. Haukaas and Terje Kalland. Prostate 3:149, 1982.
- II Effects of Diethylstilbestrol and Estramustine phosphate (Estracyt®) on lymphoid cell populations and mitogen responsiveness in male mice.
Svein A. Haukaas and Terje Kalland. J. Urol. 128:862, 1982.
- III Suppression of antibody response of male mice exposed to Diethylstilbestrol or Estramustine phosphate (Estracyt®).
Svein A. Haukaas. Prostate 4:375, 1983.
- IV Effects of Diethylstilbestrol and Estramustine phosphate (Estracyt®) on Natural Killer cell activity and tumor susceptibility in male mice.
Terje Kalland and Svein A. Haukaas. Prostate, Submitted for publication.
- V *In vitro* and *in vivo* effects of Diethylstilbestrol and Estramustine phosphate (Estracyt®) on the mitogen responsiveness of human peripheral blood lymphocytes.
Svein A. Haukaas, Per Åge Høisaeter and Terje Kalland. Prostate 4:405, 1982.
- VI Effect of treatment with Diethylstilbestrol-Polyestradiol phosphate or Estramustine phosphate (Estracyt®) on Natural Killer cell activity in patients with prostatic cancer.
Terje Kalland and Svein A. Haukaas. Invest. Urol. 18:437, 1981.

Introduction

The immune system – an overview

The immune system consists of a multitude of cell types and humoral factors which interplay in a complex manner to create an immune response. The important cells in this system are lymphocytes and macrophages. Macrophages occur in the body as tissue macrophages or circulating monocytes. These cells ingest foreign material and present it to lymphocytes. Lymphocytes circulate freely through the blood vessels and lymphatics of the body and can meet and recognize antigens whatever their localization in the host. Lymphocytes are directly responsible for all specific immune responses.

B and T cells are the two major groups of lymphocytes with distinctly different functions. They can be separated from each other by different characteristics, i.e. B and T cell markers. A small proportion of lymphocytes lacks detectable amounts of B or T cell markers and is denoted "null cells". Null cells represent a heterogeneous population that includes Killer (K) cells, Natural Killer (NK) cells as well as precursors of B and T cells which do not yet express B or T cell characteristics.

B cell function

B lymphocytes, when differentiated into plasma cells, synthesize immunoglobulins of various classes and subclasses (reviewed by Nisonoff et al., 1975). According to the clonal selection theory first outlined by Jerne (1955) and later modified by Burnet (1957), the B cell population is made up of a large number of clones, each consisting of lymphocytes carrying receptors for the same antigen and producing antibodies of the same specificity. The antibody specificity is created at the DNA level and the antigen is recognized by the B cell clones bearing complementary antibody receptors. B lymphocytes are activated by two principally different types of antigens, thymus-dependent and thymus-independent. Activation of B cells by thymus-dependent antigens requires presence of helper T cells and antigen presentation by accessory cells (Howie & McBride, 1982, Rosenthal & Shevac, 1973, Rosenthal, 1980). Thymus-independent antigens are capable of directly activating certain B cells (Andersson et al., 1972, McKearn et al., 1982).

T cell function

T lymphocytes are primarily responsible for cell-mediated immunity. The T cell repertoire is specifically directed towards transplantation antigens (products of the major histocompatible complex, MHC) from other individuals and autologous products altered by foreign antigens. This phenomenon first

described by Zinkernagel and Doherty, (1976, 1979) is called MHC restriction of T cell function. Several subpopulations exist within the T cell pool allowing a classification of helper, killer, suppressor and amplifier cells (Cantor & Boyse, 1977). These cells have distinct characteristics and do not change from one cell type to another. Their functions are to modulate immune responses in a positive or negative manner, allowing considerable variations in strength. Activation signals are a prerequisite for expression of T cell function. Certain plant lectins such as concanavalin A (ConA) and phytohemagglutinin (PHA), known as T cell mitogens, are capable of polyclonal activation of T cells with expression of all T cell functions.

Macrophages, Killer cells and Natural Killer cells

The mononuclear phagocyte system, including circulating macrophages, monocytes and tissue macrophages, have a complex role in the immune system (Nelson, 1981). In addition to effector functions such as phagocytosis and cytostasis, macrophages are required for B cell activation by most antigens and serve as accessory cells in T cell activation by releasing T cell activating factors known as interleukin-1 (Oppenheim et al., 1982). Other soluble factors released by activated macrophages, e.g. interferon and prostaglandins, modulate immune responses.

K cells and NK cells are mononuclear cells with lymphoid morphology that are capable of lysing target cells in absence of any known sensitisation. The target cells destroyed by K cells and NK cells and the cytotoxic mechanism are distinct. K cells are involved in lymphocytotoxicity against IgG coated target cells. This phenomenon is called antibody-dependent cell-mediated cytotoxicity. NK cells are capable of recognizing and killing a number of tumor cells and cells infected by virus or parasites. NK cells are presently believed to be of importance in the host defense against tumors, in particular the control of hematogenous spread of metastasizing cancer cells. Recently, NK cells have been shown to participate in the protection of the host against virus infections (for review, see Herberman, 1982).

Chemotherapy for prostatic cancer with special emphasis on estramustine phosphate

The work of Huggins and Hodges (1941), demonstrating that carcinoma of the prostate responded to androgen depletion, initiated the era of estrogen treatment for prostatic cancer. Estrogens are generally accepted as a mode of treatment for advanced cases (Scott, 1970, Barnes, 1981). Although estrogens are initially effective in slowing down the tumor growth in the majority of patients, they ultimately become ineffective (Vacarg, 1967). The management of patients with estrogen-resistant prostatic cancer has been a clinical dilemma. However, in recent years, cooperative studies on chemotherapy other than antiandrogen treatment in the management of such patients have provided evidence that the response to antineoplastic agents, such as cyclophosphamide (CY), 5-fluorouracil (5FU) and estramustine phosphate (EMP, Estracyt®) surpass the response from standard therapies (Alfthan & Rusk, 1969, Jönsson & Högberg, 1971, Mittelman et al., 1975, Murphy & Slack, 1981).

The theoretical background for the design of EMP is the attractive idea of linking a cytotoxic agent to a carrier molecule enabling specific accumulation of

the cytotoxic drug in susceptible tumor cells without causing systemic toxicity. Tissue distribution study with isotope labelled EMP, has showed retention of EMP in the human body, in which the drug can be traced by scintigraphy to the prostatic region both in normal, hyperplastic and cancerous prostates, as well as in metastasis from these neoplasms (Szendrői et al., 1973). Experimental study in the rat has demonstrated retention of EMP in the rat ventral prostate (Plym Forshell & Nilsson, 1974). The preferential accumulation in the prostate is not mediated by binding of EMP or its dephosphorylated metabolite to the classical estrogen receptor but by binding to a special prostatic protein ($K_d = 1.7 \times 10^{-8}$, maximal binding capacity = 5nmol/mg cytosol protein) (Forsgren et al., 1979, Högberg et al., 1979, Kirdani et al., 1981). Metabolic studies of EMP in man and rat have shown that EMP is rapidly dephosphorylated to give estramustine which is to a high extent oxidized to the estrone-complex, estromustine. Estromustine, one of the active metabolites, is the dominating metabolite in plasma of EMP treated patients with prostatic cancer whereas estramustine is found at higher concentration in prostatic cancer tissues than in plasma (Fritjofsson et al., 1983). Recently, distribution of EMP *in vivo* has also shown retention of EMP metabolites in spleen and thymus of the rat (Gunnarsson & Ellman, personal communications). At present, there is no settled view on the mechanism of EMP action, but some evidence exists indicating that the whole hormone-cytostatic complex may be responsible for suppression of cell proliferation in the rat prostate (Höisaeter, 1977), and for the cytotoxic effect *in vitro* on a human prostatic cancer cell line (Hartley-Asp & Gunnarsson, 1982).

Effects of estrogens on the immune system

Sex hormones are known to have important effects on the immune system. Calzolari (1898) found an increased size of thymus in male rabbits subjected to gonadectomy before puberty demonstrating for the first time a relationship between the gonads and the immune system. Exposure to estrogens, on the other hand, induce involutionary changes in the thymus (reviewed by Dougherty, 1952), loss of bone marrow and depression of the proliferation of progenitor and stem cells in the bone marrow (Urist, 1950, Boorman et al., 1980). The peripheral lymphoid tissues seem to be less sensitive to estrogens than the thymus (Kalland et al., 1978). However, some studies report a loss of lymphocytes in lymph nodes and spleen after high-dose estrogen treatment (Batchelor, 1968, Ebbesen, 1968).

There is convincing evidence for a depressive effect on cell-mediated and humoral immunity by high-dose estrogen treatment, whereas lower estrogen doses may have a stimulatory effect (reviewed by Ahlqvist, 1976, Kalland, 1980b). Females are generally believed to possess a hyperreactive antibody response as compared with males, the difference appearing at sexual maturation (Eidinger & Garret, 1972, Šljivić & Warr, 1973, Inman, 1978). The more vivid intensity of the female antibody response may be a result of both hormonal and genetic factors (Amsbaugh et al., 1972). However, inhibitory hormonal factors in the male must also be taken into consideration (Cohn, 1979). Specific binding sites for estrogens have been demonstrated in the thymus of rats and mice (Grossman et al., 1979, Gillette & Gillette, 1979), and in mouse spleen (Detlefsen et al., 1979). Recently, Danel and associates (1983) were able to identify specific estrogen binding proteins with receptor characteristics in human mononuclear blood cells, thymic cells and splenic lymphocytes. This report awaits confirmation.

Effects of alkylating agent on the immune system

Derivatives of nitrogen mustards introduced by Gilman and Philips in 1946 were the first alkylating agents in clinical use. CY is currently the most widely used drug of this class for treatment of cancer. Alkylating agents are reactive molecules and function by binding covalently to chemical groups at the level of nucleic acids and proteins. Their main biochemical impact is on DNA synthesis. Given at the time of cellular proliferation and differentiation, CY can kill a substantial number of DNA-synthesizing immunocompetent cells and may have a particularly clear immunosuppressive action.

CY is one of the most potent suppressors of antibody production. Secondary antibody response and immunological memory are also depressed although they are more resistant to CY than primary response (reviewed by Bach, 1975). Both the induction and the effector phase of delayed-type hypersensitivity (DTH) response is depressed by CY when given after antigen sensitization (Winkelstein, 1973, Turk, 1964).

Treatment with CY is reported to depress murine NK cell activity (Mantovani et al., 1978). CY sensitive NK cells in mice appear to play a significant role *in vivo* in the destruction of circulating tumor cells and in the inhibition of hematogeneous tumor metastasis (Hanna & Burton, 1981). Turk and Poulter (1972) demonstrated in experimental animals that CY *in vivo* preferentially inactivated short-lived lymphocytes. Suppressor cells are believed to have a short life-span and are sensitive to alkylating agents. CY given before antigen immunization augments immune responses such as DTH by elimination of suppressor T cells or their precursors (Miller et al., 1978). CY has also been found to reverse immunological tolerance where the unresponsiveness has been shown to be due to suppressor cells (Polak & Turk, 1974). Thus, CY is capable of interfering with the normal immunological control mechanisms by interfering with suppressor cells (Turk & Parker, 1982).

Aims of the present study

The well organized and highly regulated network of the immune system and the rapid proliferation and differentiation of its cellular constituents makes it susceptible to toxicologic effects. Cancer therapy, namely radiotherapy and chemotherapy, has major effects on the defense systems with possible implications for the defense against infections as well as the emergence and progression of cancer cells.

The wide array of tests available for specific immune functions makes the study of drug effects on the immune system a valuable and sensitive tool for evaluating drug toxicology.

Concern has been expressed that the immunosuppressive effects of high-dose estrogen therapy may add further impairment to the immune system of patients with advanced prostatic cancer already compromised by their cancer (Ablyn et al., 1976, Catalona, 1980). Moreover, information of possible effects of EMP on the immune system is severely lacking.

The aims of the present study are:

- To study in an experimental mouse system the effects of EMP and DES on cell-mediated and humoral immunity and try to reveal some of the mechanisms behind these drug effects.
- To delineate and compare possible effects of EMP and DES treatment on cell-mediated immunity in patients with cancer of the prostate.

Results of the present investigation

Effect on T cell-mediated immunity in mice (paper I)

DTH is a classical cell-mediated *in vivo* immune response as it can only be transferred by lymphocytes. Some low-molecular weight molecules such as oxazolone are able to form covalent bonds with sulfhydryl or protein amino groups present in the skin. The immunogen is a conjugate of the chemical agent and proteins of the surface epidermal cells, particularly MHC antigens of Langerhans cells.

In this study a modification of the isotopic method (i.v. injection of $^{125}\text{IUdR}$) reported by Vadas and associates (1975) was used after a challenge of oxazolone on one of both ears of sensitized mice. The immunological nature of the response measured in this assay was indicated, by the fact that in non-sensitized animals, no difference in radioactivity was found between oxazolone and vehicle (alcohol) exposed ears. Moreover, the mononuclear nature of the cellular infiltrate in experimental ears and the localization of the radioactive label to mononuclear cells was confirmed in histological preparations. Male NMRI mice at 12-14 weeks of age were treated with various doses of EMP, DES or vehicle during either the sensitization or effector phase. Treatment given for 7 days starting 2 days before sensitization and ending 5 days before a challenge with oxazolone was defined as treatment during sensitization phase. Treatment during the effector phase included 2 days prior to and including the day of challenge. Mice injected with 5.6 mg/kg DES daily showed a significantly reduced DTH response, the effect being most pronounced when given during the effector phase, reducing the DTH response to only 20% of the control value. DES induced a 50% inhibition when injected during the sensitization phase.

In contrast to DES, EMP up to 100 mg/kg/day had no significant effect if injected during the effector phase, while it induced a strong and dose-dependent depression of the DTH response when applied during the sensitization phase. The possible involvement of T suppressor cells in the DES- and EMP- induced inhibition of the DTH reaction was investigated by pretreatment with a single dose of CY 3 days before sensitization. Treatment with CY has been shown to eliminate T suppressor cells or their precursors (Miller et al., 1978). CY significantly enhanced the DTH response in control as well as in DES- or EMP-exposed animals. However, even after pretreatment with CY, a statistically significant difference existed between control and drug-exposed animals.

To further explore the possibility of suppressor cell involvement in the profound inhibitory effect of DES in the effector phase of the DTH response, spleen, lymph node and peritoneal cells from B6 mice pretreated with DES (5.6 mg/kg/day) for 7 days were adoptively transferred to syngeneic hosts sensitized with oxazolone 9 days earlier. No evidence of DES-induced suppressor cells in spleens, lymph nodes or the peritoneal cavity could be found. Moreover, no significant effects of serum transfer from DES treated animals were found in a similar system.

Effect on lymphocyte mitogenesis and lymphoid cell populations in mice (paper II)

This paper deals with the *in vitro* and *in vivo* effects of treatment with DES or EMP on mitogen responsiveness and on lymphoid cell populations of the spleen and the lymph nodes in an experimental mouse system. The ability of lymphocytes to respond to different mitogens is widely used for characterization of subpopulations of lymphocytes and as measure of general immunocompetence (Oppenheim & Rosenstreich, 1976).

Mononuclear cells were separated from whole spleen cell suspensions by the method of Böyum (1968) and the proliferation of lymphocytes cultured in the presence of various concentrations of mitogen was measured by incorporation of ^3H -thymidine. The uptake of ^3H -thymidine in a mixed lymphocyte population reflects both the responsiveness of mitogen-reactive cells and the positive and negative interactions among subclasses of lymphocytes.

Cell suspensions of spleen or lymph nodes were prepared from male B6 mice, where red cells were removed from spleen cell suspensions by hypotonic shock treatment. The enumeration of T cells and Ly subsets in spleen cell or lymph node suspensions was done using anti-Thy 1.2 antiserum and Ly-antisera in a complement-dependent cytotoxicity assay. B cells were enumerated by direct immunofluorescence using a polyvalent antibody preparation against surface immunoglobulin. Phagocytic cells were visualized after ingestion of latex particles.

The effects of both EMP and DES *in vitro* on ConA-induced mouse lymphoproliferation showed the same general picture of tending towards a slightly stimulated response at concentrations of the drugs between 0.01 and 1.0 $\mu\text{mol/l}$ and a concentration-dependent reduction of lymphoproliferation at concentration levels higher than 10 $\mu\text{mol/l}$. EMP totally abrogated the ConA response at 100 $\mu\text{mol/l}$ while DES at an equimolar concentration induced about 50% inhibition. ConA-stimulated mouse lymphocytes appeared to be more susceptible to the inhibitory effect of both DES and EMP compared to ConA-activated human lymphocytes (lymphocytes from normal young men and patients with prostatic cancer). The proliferation of LPS responsive mouse lymphocytes in the presence of various concentrations of DES or EMP was unaffected by concentrations of both EMP and DES lower than 10 $\mu\text{mol/l}$ while concentrations higher than 10 $\mu\text{mol/l}$ inhibited the proliferation.

EMP treatment of male NMRI mice resulted in a dose-related inhibition of ConA-stimulated proliferation of spleen lymphocytes compared to that of controls. The inhibitory effect of EMP treatment disappeared within 2 weeks after the last injection of EMP. In contrast to the inhibitory effect of EMP exposure on ConA response in mice, the administration of 100 mg/kg EMP daily for 7 days significantly augmented the proliferation of spleen lymphocytes to LPS whereas exposure to the lower dose of EMP was without effect on LPS response. Two weeks after the last injection the LPS response was comparable to controls.

To further delineate the mechanism behind the inhibitory effect of EMP treatment on ConA response, control spleen lymphocytes were co-cultured with Mitomycin-C (MMC)-blocked spleen cells from treated animals. In these co-cultures, about 17% inhibition was found compared to the response in co-cultures with control and MMC-blocked control spleen cells. Similar experiments were undertaken with LPS-stimulated co-cultures of control and MMC-blocked spleen cells with essentially the same result, about 21%

inhibition. Thus, we were not able to detect any significant suppressor cell activity in mitogen stimulated spleen cultures from EMP treated mice. EMP treatment of male B6 mice had a drastic effect on spleen and lymph node mononuclear cells. Ninety-four and 75% of splenic T cells were eliminated in mice exposed to 100 and 30 mg/kg EMP daily for 7 days respectively. The total number of splenic B cells and macrophages was reduced to a much lesser extent than the number of T cells and consequently these populations were relatively enriched. The T cell subset bearing the Ly 1 marker appeared to be most sensitive to EMP treatment resulting in a disproportionate T cell population.

Treatment of male NMRI mice with 1.4 or 5.6 mg/kg/day DES for 7 days inhibited the ConA response in a dose-related fashion but only the highest dose of DES reduced the response significantly. In contrast to this DES-induced inhibition of the ConA response, a biphasic response to LPS was found after treatment with 1.4 or 5.6 mg/kg/day DES. The lower dose stimulated the lymphoproliferation to LPS while 5.6 mg/kg DES inhibited the proliferation. As for the EMP treatment, the ConA response was comparable to controls 2 weeks after cessation of the treatment. On the other hand, the LPS response revealed some apparent rebound effect as the response was enhanced compared to controls at 2 weeks after the last DES injection.

The proliferation of ConA- or LPS-stimulated co-cultures of control and MMC-blocked spleen cells from DES treated mice was reduced about 16% and 2% respectively, as compared to the proliferation of control and MMC-blocked control spleen cells stimulated with the same mitogens. Hence, DES-induced suppressor cells could not be demonstrated in mitogen stimulated spleen cell cultures.

DES treatment of mice did not affect the total number of mononuclear cells in spleen and lymph nodes. However, the T cell pool in spleen and lymph nodes from DES treated mice was reduced 30% and 20% respectively. The Ly 1 subpopulation of splenic T cells appeared to be most sensitive to DES exposure as this cell population was reduced to about 50% of normal value. As a consequence of the loss of T cells in spleens of DES treated animals without change of the total number of mononuclear cells, both B cells and macrophages occurred in an increased number in these spleens.

Effect on antibody response in mice (paper III)

The effects of treatment of male NMRI mice with DES or EMP on the primary antibody response to the T cell-dependent antigen, sheep red blood cells (SRBC), and the T cell-independent antigen, bacterial lipopolysaccharide (LPS), were investigated using a direct hemolytic plaque assay for enumeration of IgM secreting cells (Cunningham & Szenberg, 1971).

Treatment of male mice with 30 or 100 mg/kg/day EMP from 2 days before to 3 days after immunization with SRBC yielded a dose-dependent reduction of the number of antibody producing spleen cells. One hundred milligram per kg body weight of EMP resulted in a significantly reduced number of IgM synthesizing cells, a decrease to about 50% of the quantity in spleens of untreated mice.

The number of anti-LPS producing cells in spleens from EMP treated animals was not significantly altered on a per 10^6 spleen cells basis, but when expressed per spleen both 30 and 100 mg/kg EMP resulted in a significantly lower response as compared with that of controls. In an initial study we found that exposure to 100 mg/kg/day EMP for 5 days before SRBC immunization

decreased the number of anti-SRBC secreting cells per 10^6 spleen cells in male B6 mice. Adoptive transfer of 10^7 T cell enriched spleen cells from EMP treated nu/+ B6 mice to the nu/nu B6 mice resulted in a significantly diminished response to SRBC as compared with the response encountered in nude mice transferred with the same number of normal T cells.

There was no significant difference in antibody response evoked by SRBC in male NMRI mice treated with 48 mg/kg/day estradiol, a dose equivalent to the amount of estradiol linked to the nor-nitrogen mustard in 100 mg/kg/day EMP. This estradiol treatment induced a significant reduction of the wet weight of the seminal vesicle indicating a significant effect on a sex hormone sensitive target organ (unpublished observation). Exposure to 1.4 or 5.6 mg/kg/day DES for 7 days resulted in a significant reduction of the anti-SRBC as well as the anti-LPS response except that the lower dose had a slight, but insignificant, effect on the number of anti-LPS producing cells on a per spleen basis.

Effect on mouse NK cells and tumor susceptibility (paper IV)

In this paper the effects of treatment with DES or EMP for 6 days in male B6 mice on the *in vitro* NK cell activity of spleen cells were investigated. Splenic NK cell activity against YAC-1 target cells (a NK-sensitive cell line derived from a Moloney virus-induced lymphoma of A/Sn origin) was measured in a short-term $^{51}\text{Chromium}$ (^{51}Cr) release assay modified to allow calculation of kinetic parameters for NK activity (Callewaert et al., 1982, 1983). NK activity was assessed at the single cell level by plating of the NK cell-target-cell conjugates in agarose gel giving information of both target cell recognition of NK cells and the subsequent lysis of the target cell (Targan et al., 1980). The susceptibility to Lewis Lung Carcinoma (LLC) was studied in DES or EMP treated syngeneic B6 mice by inoculation subcutaneously (s.c.) of 5×10^3 cells or injection intravenously (i.v) of 10^5 cells of a freshly prepared single cell suspension from *in vivo* passed LLC tumors. The syngeneic LLC cells were shown to be sensitive to NK mediated cytotoxicity, although somewhat less sensitive than the standard target YAC-1.

Treatment with 100 mg/kg/day EMP for 6 days decreased NK activity 24 hours after the last injection. The number of target-binding NK cells in the spleen was found to be normal while their lytic capacity was impaired. The EMP-induced inhibition of NK activity could be reversed by a single intraperitoneal injection with 100 μg polyinosinic: cytidylic acid (Poly I:C), an interferon inducer, simultaneously with the last injection of EMP. The inhibitory effect of EMP vanished within 7 days after cessation of treatment. Male B6 mice given 5.6 mg/kg DES daily for 6 days showed an altered NK activity in the spleen 24 hours after the last injection. In contrast to the EMP treatment, the effect of DES was a reduced number of NK cells able to bind their target while the lytic activity and recycling capacity was augmented. Poly I:C treatment, as described earlier, had no boosting effect on NK cell activity. Just as with the EMP treatment, the DES-induced perturbation of NK activity was transient and NK activity normalized within 2 weeks after the last injection.

Treatment of mice with either 100 mg/kg/day EMP or 5.6 mg/kg/day DES for 6 days resulted in an earlier appearance and increased incidence of palpable tumors in treated mice as compared with controls. Control animals developed about 30 % palpable tumors at 40 days from inoculation while about 60 % of

the mice in the 2 treatment groups had tumors at the same point of time. The number of lung tumors arising in EMP exposed mice after i.v. injection of LLC cells was significantly increased compared to the number of tumors in lungs of control animals. DES treated mice, however, had numbers of lung tumors comparable to control animals.

***In vitro* and *in vivo* effects on human T cell mitogenesis (paper V)**

The mitogen-reactivity to the T cell mitogens, ConA or PHA, of peripheral blood lymphocytes from 6 healthy young men and 18 prostatic cancer patients aged 61–79 years was tested in cultures supplemented with various concentrations of DES or EMP (0.12 – 2.4 mmol/l). Mononuclear cells were obtained from peripheral blood (Böyum, 1968) and lymphoproliferation was measured after a 72 hour incubation with mitogens as the uptake of ^3H -thymidine added to the culture 20 hours before its termination.

The response of ConA-stimulated lymphocytes from healthy young men and from patients with various stages of prostatic cancer before treatment showed the same trend, i.e. a concentration-dependent inhibition at EMP concentrations higher than 0.12 mmol/l or DES concentrations higher than 0.24 mmol/l. Furthermore, EMP strongly inhibited PHA-stimulated proliferation at concentrations above 0.12 mmol/l, while DES at the same concentrations had no inhibitory effects on PHA-induced proliferation. Hence, EMP proved to be a more potent inhibitor of ConA- and PHA-induced lymphoproliferation than DES on an equimolar basis. Peripheral blood lymphocytes from patients treated for 4 weeks with DES or EMP showed the same degree of inhibited proliferation by DES or EMP *in vitro* as before treatment.

The *in vivo* effect of oral treatment with EMP, conventional doses of DES or high-dose i.v. DES diphosphate on mitogen responsiveness of peripheral blood lymphocytes was investigated in patients treated for prostatic cancer. Oral treatment with 840 mg EMP daily for 4 weeks was given to 7 patients. All patients were tested for mitogen response to ConA before and after treatment, but only three patients were assayed for PHA responsiveness. No significant alterations were observed. However, there was considerable variation in the individual values of relative proliferation before and after treatment. Peripheral lymphocytes from one patient showed a substantial augmentation of ConA response, while lymphocytes from the majority of patients responded less well after EMP treatment. The serum source, either autologous or homologous (pooled AB serum), appeared to be without significant influence on the mitogen response.

The mitogen responsiveness of peripheral blood lymphocytes was not significantly altered after oral treatment with 10 mg DES for 2 weeks and later 2 mg daily, supplemented with 80 mg polyestradiol phosphate injected intramuscularly (i.m.) on the first day of treatment. On the other hand, 1000 mg DES diphosphate administered i.v. daily for 1 week inhibited PHA-stimulated proliferation, but ConA responsiveness was unaltered after the same treatment.

Effect on human NK activity (paper VI)

The NK activity in peripheral blood of patients with prostatic cancer ranging from 61-79 years of age was measured in an *in vitro* cytotoxicity assay. Mononuclear cells were obtained from peripheral blood by the method of Böyum (1968) and cultured for 4 hours together with ^{51}Cr -labelled Molt-4 target cells (Molt-4 is a human T-lymphoblast cell-line). The cytotoxicity was expressed as per cent specific ^{51}Cr -release from target cells.

In this study an evaluation was made of the effect on NK cell activity in peripheral blood in prostatic cancer patients after oral treatment with 840 mg EMP daily, or the combination of oral therapy with DES (10 mg daily for 2 weeks and later 2 mg daily) and polyestradiol phosphate (80 mg i.m.) on the first day of treatment. NK activity during treatment was measured at 1 and 4 weeks after initiation of therapy and compared to the pretherapy NK activity, i.e. each patient being his own control.

EMP apparently did not influence NK activity, as the level at 1 and 4 weeks after initiation of therapy was the same as the pretherapy level. Estrogen treatment consisting of a combination of DES and polyestradiol phosphate substantially reduced NK activity after a treatment period of 1 week, and the activity was further slightly decreased 3 weeks later. The 2 patients given high-dose i.v. DES diphosphate (1000 mg daily) had a decreased response when therapy was changed after 1 week. The small number of patients included in this study did not allow evaluation of a possible dose-response relationship of DES on NK activity. However, the results indicate that DES-polyestradiol phosphate and high-dose DES treatment significantly reduced NK activity compared to pretherapy levels and this in contrast to treatment with EMP.

General discussion

The present study demonstrates that DES is a potent inhibitor of mitogen-induced proliferation of mouse spleen lymphocytes *in vitro* at concentrations higher than 10 $\mu\text{mol/l}$. Furthermore, mouse lymphocytes have been shown to be more susceptible to inhibition *in vitro* by DES than human peripheral blood lymphocytes as only 50 % inhibition occurred in human lymphocyte cultures stimulated with T cell mitogens even at the highest concentrations of DES (papers II, V).

Sex hormones are generally found to inhibit mitogen-stimulated proliferation of lymphocytes *in vitro* at pharmacological concentrations (Ablin et al., 1974, Harty et al., 1976, Wyle & Kent, 1977, Mendelsohn, 1977). This evidence argues against a specific, receptor-mediated immunosuppressive action *in vitro* of sex hormones. Moreover, estrogen receptors have not been convincingly demonstrated in resting or lectin-stimulated lymphocytes (Neifeld et al., 1977), although Danel et al. (1983) claim to have demonstrated estrogen binding sites in human lymphoid cells.

Homo and colleagues (1980) suggest that sex hormones probably act in a nonspecific manner at the level of the cell membrane. These authors could not demonstrate any cross-reactivity between sex hormones and glucocorticoid receptors in lymphocytes. Alterations in calcium flux through the lymphocyte membrane have been suggested to be an early event in mitogen-induced proliferation and estrogens may prevent the entry of calcium into lymphocytes (Whitfield et al., 1973, Morgan & Perris, 1975). Moreover, estrogens also inhibit glucose and nucleoside transport in various cells and thereby interfere with normal cell functions (Plageman & Renner, 1972, Homo et al., 1980). A colchicine-like effect on microtubuli of high concentrations of DES has been described (Sawada & Ishidate, 1977) and may offer an explanation for inhibition of lymphoproliferation.

In the present study EMP is shown to inhibit the mitogen-induced proliferation of mouse spleen lymphocytes *in vitro* at concentrations above 10 $\mu\text{mol/l}$. A tenfold higher concentration of EMP is required to inhibit the proliferation of human peripheral blood lymphocytes stimulated with mitogens. EMP appears to be a more potent inhibitor of lymphocyte mitogenesis than DES on an equimolar basis (papers II, V).

The mechanism behind the inhibitory effect of EMP on mitogen-induced lymphoproliferation *in vitro* is not clear. There is evidence that the intact estramustine molecule is responsible for the inhibition of proliferation and clonogenic survival of hormone independent prostatic cancer cells in continuous culture (Hartley-Asp & Gunnarsson, 1982). Besides, EMP as well as nor-nitrogen mustard have a marked inhibitory effect on ^3H -thymidine incorporation in explants of rat ventral prostate (Höisaeter, 1977).

Human lymphocytes cultured for 72 hours in the presence of ConA and estramustine metabolized about 4 % of the total added estramustine, yielding free estradiol and nor-nitrogen mustard. Estramustine, and to a lesser degree estromustine, but no estradiol, were detected in the cell pellet from these cultures. Estramustine, estradiol and nor-nitrogen mustard added to ConA-stimulated human lymphocyte cultures showed comparable inhibition of lymphoproliferation (Gunnarsson et al. unpublished observations), thus the concentrations available in ConA-stimulated lymphocyte cultures of the separate components of estramustine are too low to account for the inhibition. The most attractive interpretation of these data is that the intact estramustine

molecule in some way inhibits lymphocyte mitogenesis *in vitro*.

Independently of whatever the mechanism of action of estrogens and EMP is on the single cell level, the overall effect on lymphoproliferation may be a result of interference with subpopulations of cells in the cultures required for a normal proliferative response to lectins. Results from our laboratory indicate that EMP interferes with the production or release of interleukin-2 (IL-2), a T cell growth factor produced by T helper cells, in lectin-stimulated mouse and human lymphocytes *in vitro* (Kalland & Haukaas, 1983).

Treatment of patients with a conventional regimen of DES-polyestradiol phosphate for prostatic cancer does not induce any detectable impairment of T cell proliferation to ConA or PHA while high-dose treatment depresses PHA-stimulated proliferation of T cells (paper III). Ablin and associates (1976, 1979) reported decreased PHA responsiveness of lymphocytes from patients treated with DES. However, the exact treatment regimen was not given in these studies. The relation between DES concentrations able to inhibit the T cell response to mitogen *in vitro* and the actual serum levels reached during treatment is of considerable interest. Abraham and associates (1972) have reported peak serum level of DES of 10 nmol/l after the ingestion of a single 10-mg dose, while Guinan and associates (1975) estimate a serum level of 100 nmol/l after an oral dose of 5 mg DES. Although the measurement of DES serum levels are divergent, a concentration level sufficiently high so as to perturb mitogen responsiveness is probably only reached during high-dose i.v. DES therapy. However, various components of the immune response may show different sensitivity towards DES. NK cells of patients with prostatic cancer are apparently more sensitive to DES *in vivo* than T cells as NK activity was markedly reduced in patients during DES therapy whereas T cell proliferation to mitogens was undisturbed (paper VI).

In the mouse model DES induced no alteration of overall NK activity but a further analysis by stimulation with Poly I:C and by using a single cell assay in agarose disclosed subtle perturbations of different parts of the NK cell response. The number of target-binding NK cells in spleens of DES treated mice is reduced but, on the other hand, the lytic ability and recycling capacity is augmented resulting in a normalization of NK cell activity (paper IV). Estrogens, DES inclusive, are well known stimulators of macrophages (Vernon-Roberts, 1969) and the stimulation of lytic activity and recycling capacity could be suggestive of an increased production of interferon from DES activated macrophages.

Short-term, high-dose DES treatment of mice has been shown to induce bone marrow hypocellularity and to inhibit the proliferation of bone marrow progenitor cells and stem cells. The estrogen-induced inhibition of bone marrow cell proliferation is prevented by simultaneous administration of tamoxifen or prethymectomy (Boorman et al. 1980, Luster et al., 1983). The reduced number of target-binding NK cells in spleens of DES treated mice is in keeping with an inhibitory effect on the bone marrow as NK cells are continuously renewed from bone marrow (Haller & Wigzell, 1977, Miller, 1982). In experimental studies, Seaman and associates (1978, 1979 a,b) observed that continuous treatment with estradiol for 6 weeks substantially reduced NK activity in the spleen along with new bone formation encroaching on the bone marrow in mice. NK activity could not be boosted by treatment with Poly I:C. The loss of NK activity may reflect a loss of bone marrow secondary to estrogen-induced osteosclerosis.

NK cell activity is shown to be unaltered in peripheral blood of patients treated with EMP for prostatic cancer (paper VI). The result may indicate a

low bone marrow toxicity of EMP as human NK cells seem to be dependent on the bone marrow for normal maturation. Hematological parameters have been reported to be within normal range in patients treated with EMP indicating that EMP causes few overt bone marrow side effects (Jönsson & Högborg, 1971, Murphy & Slack, 1980). Furthermore, EMP appears to be rather harmless to human T cells *in vivo* (paper V). Küss and associates (1980) found no effect on the DTH response to several recall antigens in patients with prostatic cancer supporting our findings that EMP treatment induce no detectable impairment of cell-mediated immunity.

In the study of mouse NK activity, kinetic analysis was applied. Several authors have compared this approach with the standard assay and found the former to give a better estimate of the lytic activity of a given effector cell population (Callewaert et al, 1978, Pollack & Emmons, 1979, Van Oers et al, 1978, Ullberg & Jondal, 1981).

Based on the Michaelis-Menten model for enzyme-kinetics, the NK cell - tumor cell interaction can be expressed in the following way:



Given certain experimental conditions, this reaction will approximately follow the Michaelis-Menten equation where the relationship between the concentration of substrate (tumor cells) and the speed of the reaction is:

$$V = \frac{V_{\max} \times (S)}{(S) + K_M}$$

where V is the velocity of product formation (killed tumor cells), (S) the concentration of substrate and K_M the Michaelis-Menten constant. Interpretation of the K_M value in the present system is a matter of controversy and has recently been suggested to reflect the percentage of lytically active NK cells (Callewaert et al, 1983).

In contrast to the resistance of human NK and T cells to EMP treatment, cell-mediated immunity in the mouse is severely depressed by EMP treatment. The inhibition of NK cell activity evidenced by the reduced $V_{\max}$ value could be reversed by treatment with Poly I:C, an agent shown to stimulate macrophages to production of interferon. Interferon is said to be the most important regulator of NK activity *in vivo* and the effect is described to be recruitment of active NK cells from inactive precursors, stimulation of lytic capability of individual NK cells together with an enhancement of recycling capacity, i.e. the number of target cells killed by a single NK cell (Targan & Dorey, 1980, Ullberg & Jondal, 1981). Hence, it is possibly that lytic active NK cells are impaired by EMP treatment while precursor NK cells are resistant to EMP and are inducible by interferon into active NK cells. Alternate possibilities are that interferon is able to stimulate the lytic machinery of EMP impaired NK cells enough to overcome the inhibition or that interferon stimulates an EMP resistant subpopulation of NK cells in a sufficient degree to restore lytic activity.

EMP treatment in male mice has been shown to induce thymic involution as thymic weight was reduced in a dose-dependent manner and routine histology revealed increased cortical degeneration (Kalland & Haukaas, 1983). In accordance with this finding, EMP treatment of mice has a preferential effect on thymus-dependent immune function. EMP has a differential effect on ConA- and LPS-induced lymphoproliferation in mice as the lectin-stimulated T

cell proliferation is depressed while the proliferation of B cells is augmented. The seemingly stimulated B cell response is probably related to a relative enrichment of B cells in spleens of EMP treated mice (paper II).

The preferential depletion of T cells, in particular T helper cells, in spleens and lymph nodes of EMP treated mice indicates that these cells are the main targets of EMP (paper II). T helper cells play a central role as inductor or amplifier cells in most immune responses. Impairment of these cells will be reflected in a wide variety of immunological assays. The present data from the mouse studies on immunological effects of EMP treatment are compatible with a defect T helper cell function (papers I, II, III). Recently, results from our laboratory demonstrate that EMP interferes with IL-2 production, or release, in lectin-stimulated lymphocytes *in vitro* (Kalland & Haukaas, 1983). IL2, a lymphokine produced by T helper cells, is a prerequisite for T cell proliferation. A tentative EMP-induced inhibition of IL-2 production *in vivo* could to a large extent explain the immunosuppressive effects of EMP seen in the mouse. However, there is at present no direct evidence supporting this suggestion.

The present experimental results indicate that acute exposure to high dose DES in mice induces impairment of both T cell and B cell associated functions. The depression of ConA and LPS responsiveness of spleen lymphocytes point to an antiproliferative effect of DES. Evidence based on further analysis of the cell constituents in spleens from DES treated mice compared with spleens from untreated animals indicates that DES induces disturbances in the T cell population with loss of the T cell subclass responsible for induction and helper function in the immune response. The impairment of T cell function is suggested to be one of the main effects of DES on the immune response, as evidenced by reduced DTH reactivity, inhibited ConA response and depression of antibody response to T cell-dependent antigen (papers I, II, III). In keeping with this concept are the results from studies on immune effects of neonatal exposure to DES (Kalland 1980b).

Luster and associates (1980) provide evidence that DES-induced inhibition of mitogen-stimulated lymphoproliferation can be related to suppressor cells residing in the macrophage population of the DES treated mice. Depletion of adherent cells from spleens of DES treated mice resulted in an enhancement of the ConA response compared to the response using lymphocytes from untreated mice. Co-culture of macrophage depleted spleen cells with 10% adherent peritoneal macrophages from DES treated mice yielded 40% inhibition of ConA response compared to controls using normal macrophages (Luster et al., 1980). However, we were not able to find any significant depression of the ConA response when normal spleen cells were co-cultured with MMC-blocked spleen lymphocytes from DES treated mice (paper II).

The inhibition of DTH response induced by DES is most pronounced when given during the effector phase (paper I). Luster and associates (1980) reported a depressed DTH response in mice when DES was injected after protein antigen sensitization whereas the DTH response was comparable to control when DES preceded sensitization. These authors conclude that the immunosuppressive effect of DES appears to have a restricted persistence, but an effect on memory cell function cannot be readily dismissed (Luster et al., 1980). In our experimental model of the DTH response we were not able to find any suppressor cells or humoral suppressor factor responsible for the depression induced by DES (paper I). One can also speculate if the effect of DES in the effector phase of the DTH response can be related to indirect effects such as alteration in the connective tissue. Estrogens influence the

vascular ground substance, rendering it less permeable (Müller-Beissenhirtz et al., 1971) and may thus interfere with the locomotion of effector cells to the site of antigen application. This effect will also decrease the availability of lymphokines and is consistent with the preferential action of DES in the effector phase.

Šljivić and Warr (1973) suggest that the DES-induced depression of the antibody response can be related to a stimulatory effect on the reticulo-endothelial system (RES), causing diversion of antigen from the site of initiation of the response. A RES related estrogen effect on the antibody response, however, can be questioned because DES inhibits the antibody response to LPS, one of the least macrophage-dependent antigens known (Chused et al., 1980). The depressive effect of estrogens on antibody production seems to occur only when high doses of estrogens are given before immunization (Batchelor & Chapman, 1972, Toivanen, 1967, Šljivić & Warr, 1973, Ezaki et al., 1982). Kenny and associates (1971) demonstrated a dualistic effect of estradiol *in vitro* on proliferation of immunocompetent cells, i.e. low concentrations enhanced, whereas high concentrations of estradiol inhibited the proliferation of antigen sensitized lymphocytes. Paavonen et al. (1981) reported that physiological concentrations of estradiol had an enhancing effect on the antibody response in pokeweed mitogen stimulated human lymphocyte cultures by inhibiting T suppressor cells. Higher concentrations of estradiol inhibited the proliferation and differentiation of B cells. Evidence from the present study (paper II) and from the study of Luster et al. (1980) support this concept of a dualistic effect on B cell proliferation. However, the differentiation of B cells to IgM producing cells is shown to be depressed in a dose-dependent manner after mice are treated with the same DES dose (paper III, Luster et al. 1980).

The present study also disclosed a divergent effect of the two estrogens DES and estradiol on the antibody response, as a high dose DES inhibited it, while a high dose estradiol was without effect (paper III). Other investigators have found that estradiol at high doses inhibit the antibody response although sex and species differences together with variations in immunization and treatment procedures make the comparison of results difficult. The conflicting effects on antibody production of the high doses estradiol and DES reported in Paper III, may indicate that beyond being a potent estrogen, DES or DES-metabolites possess immunosuppressive properties independent of an estrogen receptor-mediated mechanism. However, this assumption is questioned by recent observations by Luster and associates (1983) that the estrogen antagonist clomiphene partially reverses DES-induced impairment of the immune response. On the other hand, the antiestrogenic properties of clomiphene have not been convincingly demonstrated in the mouse (Nicholson & Griffiths, 1980).

Ample experimental evidence indicates that NK cells play an important role *in vivo* in the destruction of circulating tumor cells and in the prevention of spontaneous metastatic spread of tumor cells (Kasai et al., 1979, Talmadge et al., 1980, Kärre et al., 1980). Chronic exposure to estradiol, known to inhibit NK cells in mice, resulted in a prolonged survival of tumor cells and was associated with an increased incidence of experimental and spontaneous pulmonary metastasis of the tumors in mice (Hanna, 1982). The impairment of NK cells, as demonstrated in EMP treated mice, may provide a plausible explanation for these animals increased susceptibility to syngeneic tumor cells. DES treated mice with a demonstrably reduced number of active NK cells in the spleen, yet showing an increased killing efficiency in the remaining NK

cells, had no demonstrable increase of lung tumors following i.v. injection of syngeneic tumor cells as compared to control animals. These results further support that there is a close relation between levels of host NK activity and the capacity to eliminate tumor cells from the blood stream. On the other hand, DES treatment rendered mice more susceptible to tumor growth after s.c. implantation of syngeneic tumor cells. It is possible that NK cells in different locations in the host have different sensitivity to DES. However, other host defense mechanisms with differential sensitivity to DES may be active against tumor growth after s.c. injection of tumor cells, e.g. T cells or macrophages. Dean et al. (1980) proposed that T cell dysfunction found in DES exposed mice might account for altered tumor resistance.

The present investigation provides evidence that both EMP and DES have immunosuppressive effects which affect the outcome of tumor growth or metastasis in mice. The patient study indicates that cell-mediated immunity is not impaired by commonly used doses of EMP while NK cell activity is markedly reduced in DES treated patients. High-dose DES therapy also inhibits human T cell proliferation with PHA. In experimental animals, there is evidence that high levels of NK cell activity have a protective role against metastasizing tumor cells, and protect against infections with virus or parasites. However, the biological role of NK cells in control of metastasis or infections in tumor-bearing patients is unknown. Whether the differential effect of EMP and DES on NK cell activity has any consequence for control of metastasis in patients with prostatic cancer has to be established.

Conclusions

Exposure of mice to EMP had a marked, though transient, suppressive effect on T and B lymphocyte associated functions. Evidence from different immunological assays indicated that T lymphocytes, in particular T helper cells, were the preferential target for EMP. NK cell activity in spleens of EMP treated mice was reduced by an interference with NK lytic events. EMP treatment of mice impaired the host resistance to transplanted tumor cells.

High-dose DES treatment of mice induced a transient impairment of immune functions mediated by T and B lymphocytes. DES perturbed the balance between different subpopulations of T cells by reducing the T helper subclass, and also inhibited the differentiation of B cells to antibody-producing cells. DES interfered with the differentiation of precursor NK cells to target-binding NK cells, but also stimulated their killing capacity resulting in a normalization of splenic NK activity. DES treated mice had a normal capacity to eliminate circulating tumor cells, but had a defective host defense against s.c. transplanted tumor cells.

Mouse lymphocytes were generally more susceptible to the immunosuppressive effect of both EMP and DES than human lymphoid cells. Peripheral blood lymphocytes from EMP treated patients showed unaltered mitogenesis to T cell mitogens and an unchanged NK activity. On the other hand, NK activity was markedly decreased in DES treated patients. High-dose DES treatment reduced the PHA-induced proliferative response of peripheral blood lymphocytes from patients. The clinical implications of the different effects of EMP and DES on NK activity in humans remains to be established.

Acknowledgements

The present study has been carried out at the Institute of Anatomy, University of Bergen, and at the Department of Anatomy, University of Lund, during the years 1979 – 1983.

I wish to express my gratitude to:

Professor John-Gunnar Forsberg, who initiated this work, for his encouragement and generous support and for providing me with excellent working facilities,

Assistant professor Terje Kalland for introducing me to the field of immunology and for invaluable help, advice and criticism,

Senior surgeon Thorolf Gjersvik and professor Per Åge Höisäter, Department of Surgery, Urology section, University of Bergen, for never failing interest and support,

Professor Torsten Sundin, Department of Urology, University of Lund, for interest and support,

My former colleagues at the Institute of Anatomy, University of Bergen, for their help and advice,

Mrs. Erna Finsås, Åse Flatnes, Gry Kvalheim, Nina Lied Larsen and Kerstin Nilsson for skilfull technical assistance,

Mrs. Agneta Persson, Miss Liv Skarstein and Mr. Ragnar Jensen for doing the drawings and the photographical work,

Mrs. Kirsten Mehl, Mrs. Christina Parknäs, Karin Weltzin and Miss Ann-Christin Persson for secreterial assistance,

Mr. Michael Falman for revising the English text.

This study was supported by grants from The Swedish Cancer Society (Project 1870-B83-01XA), AB Leo Research Foundation, Helsingborg, The Medical Faculty at the University of Lund, The Norwegian Cancer Society, The Norwegian Council for Science and the Humanities and The Medical Faculty at the University of Bergen.

Permission to include papers in the Appendix section of this volume was generously granted by Alan R. Liss, Inc., New York and the Williams and Wilkins Co., Baltimore.

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Appendix: Papers I—VI

Effect of Diethylstilbestrol and Estramustine Phosphate (Estracyt®) on Delayed Hypersensitivity Response to Oxazolone in Male Mice

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The effects of diethylstilbestrol (DES) and estramustine phosphate (EMP) on delayed hypersensitivity response (DTH) to oxazolone in male mice were investigated using a radioisotopic ear method. DES significantly inhibited the ability to express a DTH response and was most effective when given during the effector phase of the reaction. EMP had no effects when the animals were exposed to the drug during the effector phase, while it resulted in a strong and dose-dependent depression of the DTH response when applied in the sensitization phase. Possible involvement of suppressor cells was investigated by pretreatment of animals with cyclophosphamide or by adoptive transfer of tentative suppressor cells from spleen, lymph nodes, or peritoneal cavity. However, no evidence of cellular or humoral suppressor factors induced by drug treatment was found.

Key words: diethylstilbestrol, estramustine phosphate, delayed hypersensitivity response

INTRODUCTION

Since the initial report by Huggins [1], estrogenic therapy has gained general acceptance as the mode of treatment of advanced prostatic cancer [2]. The currently most applied agent is the nonsteroidal estrogen diethylstilbestrol (DES). Recently, concern has been raised as to whether the beneficial effects on the tumor may be counteracted not only by the well-known cardiovascular side effects of estrogens [3, 4], but also by DES-induced disturbances in the immune response [5–7]. In patients treated for cancer of the prostate, DES has been shown to inhibit mitogen-induced lymphocyte proliferation, tumor-specific leukocyte adherence inhibition, and natural killer cell activity (8–10). In rodents, DES stimulates the reticuloendothelial system [11], while DES exposure of the neonatal female mouse leads to persistent disturbances of several aspects of the immune response, including delayed hypersensitivity [12–14].

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The steroidal-alkylating agent estramustine phosphate (EMP, Estracyt®) has been recommended as an alternative to estrogens in the treatment of poorly differentiated carcinomas of the prostate [15, 16]. Information on possible effects of EMP on the immune system is severely lacking, although a recent comparative study on the effects of DES and EMP on the NK activity in patients with prostatic cancer revealed that EMP in contrast to DES did not affect NK cells [10]. Moreover, Küss et al [17] did not detect alterations in delayed hypersensitivity response to recall antigens during treatment with EMP.

The present investigation was undertaken to further delineate and compare the effects of DES and EMP on the immune system.

MATERIALS AND METHODS

Animals

All mice used were males belonging either to a randomly bred NMRI stock kept at our institute or to the inbred strain C57b1/6 (B6) purchased from Glaxo Børnholthgård, Denmark. They were fed a standard pellet diet and given water ad libitum. The animals were used at 12–14 wk of age and had a mean weight of 35 ± 5 gm (NMRI) or 25 ± 5 gm (B6). The animals were exposed to the drugs under investigation during the different phases of the delayed hypersensitivity (DTH) response (see Results).

Experimental Procedure

The delayed hypersensitivity test was performed as a modification of the radioisotopic method reported by Vadas et al [18]. Briefly, the animals were sensitized by two applications of 100 μ l 10% 4-ethoxy-methylene—phenyl oxazolone (BDH Chemicals Division, Poole, England) at a 24-hr interval. Oxazolone was dissolved in ethanol and applied on an area of shaved abdominal skin. Nine days after sensitization the animals were painted on the dorsal surface of the right ear with 50 μ l 3% oxazolone and on the left ear with the solvent only. Ten hours after challenge, an intravenous pulse of 0.1 ml saline containing 2 μ Ci 125 Iododeoxyuridine (125 IUDR, specific activity 5 Ci/mg, The Radiochemical Center, Amersham, England) was given into the tail vein. Sixteen hours after isotope injection the mice were killed, the ears cut off, placed in plastic tubes, and counted in a Searle 1185 gamma spectrophotometer. The results were expressed as the logarithm of the difference in radioactivity between the right and left ears and the effect value of the experimental groups related to the effect value of control animals.

In some experiments, the sensitization procedure was omitted to ensure the immunological nature of the response. Some preparations were processed for autoradiography as described earlier [19].

Elimination of Suppressor Cells

To eliminate T suppressor cells a single IP injection of 100 mg/kg cyclophosphamide (CY) was given 3 days prior to sensitization with oxazolone [20]. This dose was in initial experiments found to be optimal in increasing the DTH response.

Adoptive Transfer System

B6 mice pretreated for 7 days with 5.6 mg/kg/day DES were killed by cervical dislocation and their spleen and lymph nodes rapidly dissected out. Single-cell suspensions were made up in phosphate-buffered saline (PBS, pH 7.4). Red cells were removed from

the spleen-cell suspensions by hypotonic shock treatment and 2×10^7 spleen cells or 5×10^6 lymph-node cells in 0.1 ml PBS were then injected into the tail vein of mice sensitized with oxazolone 9 days earlier. Control animals received cells from animals pretreated with olive oil. The animals were challenged with oxazolone and assayed for DTH response as described above.

Peritoneal macrophages from mice pretreated with 5.6 mg/kg/day DES were harvested by aspiration of 4 ml PBS 5 min after its installation in the peritoneal cavity. In a similar system as described above, 5×10^5 macrophages were transferred IV to host animals. Blood drawn from the orbital plexus of animals pretreated with either DES or olive oil were allowed to clot at room temperature and 0.5 ml of the serum were injected IV to oxazolone-sensitized animals according to the same schedule as described for cell transfer. The animals were then tested for DTH response to reveal possible humoral suppressor factors induced by DES.

Statistical Methods

The difference in cpm value between the test and control ear of the same animal was found to be skewly distributed, but after a logarithmic transformation a normal distribution was obtained and further used for all statistical evaluations. Results for the different treatment groups were compared by using Fisher's variance analysis or Student's *t* test. Before doing these comparisons it was checked that the error variances of the groups involved did not differ significantly. When more than two groups were compared this was done by using Bartlett's test for homogeneity of variances [21].

Significance levels were $0.05 < P < 0.01^*$, $0.01 > P > 0.001^{**}$, $P < 0.001^{***}$, and ns, not significant.

RESULTS

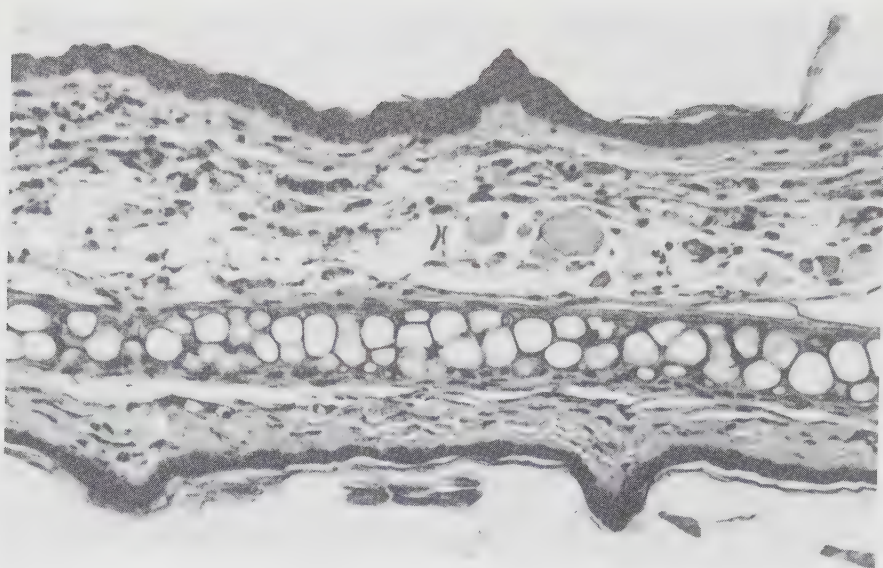
The immunological nature of the response measured in this assay is indicated from the fact that in nonsensitized animals, no difference in radioactivity was found between oxazolone and vehicle-exposed ears. Moreover, the mononuclear nature of the cellular infiltrate in experimental ears and the localization of the radioactive label to mononuclear cells was confirmed in histological and autoradiographic preparations (Fig. 1).

The effect of exposure to DES or EMP either during the sensitization or the effector phase of the DTH response to oxazolone in male NMRI mice is shown in Figure 2. The highest dose of DES significantly reduced the DTH response, the effect being most pronounced in the effector phase reducing the DTH response to only 20% of the control value. DES induced a 50% inhibition when injected during the sensitization phase.

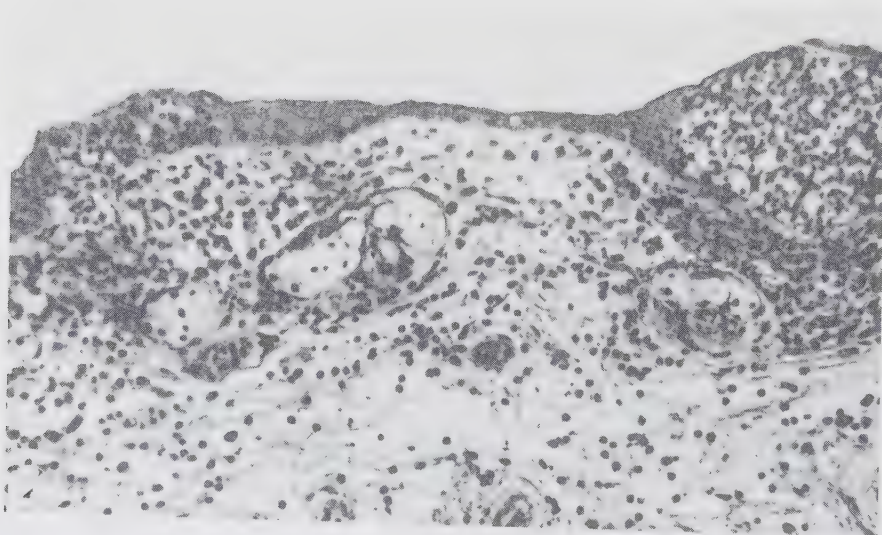
In contrast, EMP had no significant effects when given during the effector phase while it resulted in a strong and dose-dependent depression of the DTH response when applied during sensitization. EMP daily (100 mg/kg) reduced the ability to mount a DTH reaction to about 25% while 30 mg/kg gave a 60% response compared to control values. It should be noted that 30 mg/kg EMP was somewhat stimulatory when given during the effector phase although this difference was not statistically significant.

Some studies on the mechanism of the inhibition of the DTH response were performed with B6 mice and Table I contains results from parallel studies as described above in this mouse strain showing essentially the same trend as with NMRI mice.

The possible involvement of T suppressor cells in the DES- and EMP-induced inhibition of the DTH reaction to oxazolone was investigated by elimination of suppressor cells with Cy. The effect of pretreatment of animals with Cy before exposure to DES or



a



b

Fig. 1. (a). Histological section through the control ear (painted with vehicle only) of a mouse sensitized with oxazolone 9 days earlier. Haematoxylin-eosin staining. $\times 160$. (b). Histological section through the experimental ear (oxazolone painted) from the same animal as in (a). Note the dense mononuclear cell infiltration. $\times 180$.

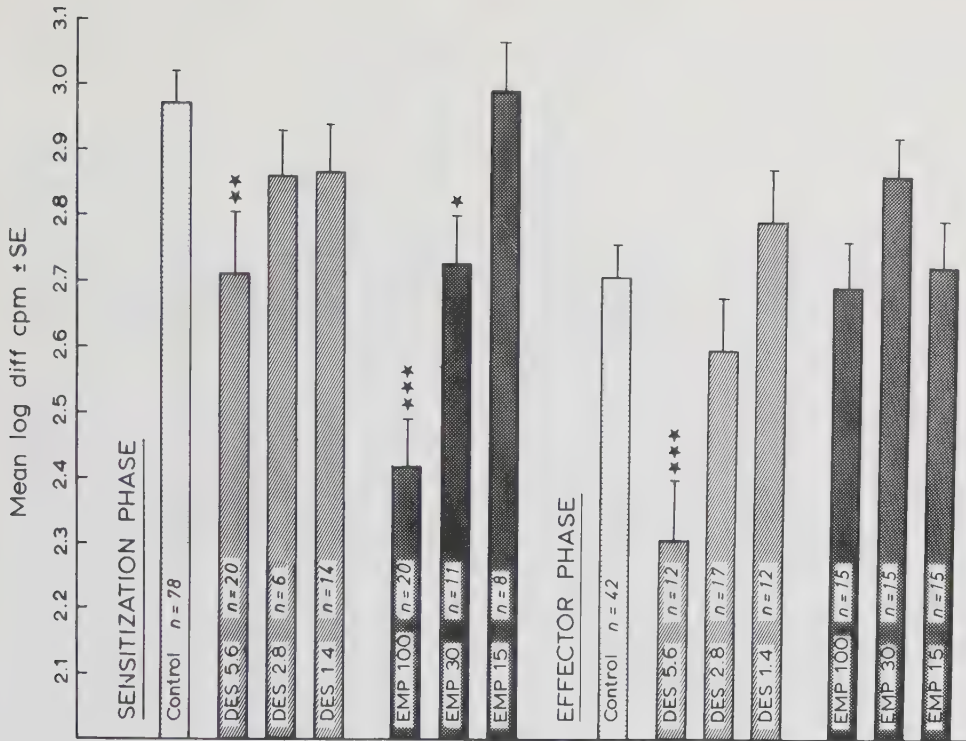


Fig. 2. Effect of exposure to various doses of DES or EMP during the sensitization or effector phase of a delayed hypersensitivity response. Treatment during sensitization includes 2 days before as well as 3 days after sensitization. DES was given as a single sc injection, EMP as 2 daily ip injections. Treatment during the effector phase includes 2 days prior to and the same day as challenge was performed. n = number of animals in each group. The asterisks indicate statistical significance as defined in Materials and Methods.

EMP is shown in Table II. Cy significantly enhanced the DTH response in control as well as in DES-exposed animals. However, even after pretreatment with Cy, a statistically significant difference existed between control and DES-exposed animals. The same conclusions were reached when Cy was combined with EMP. To further explore the possibility of suppressor-cell involvement in the profound inhibitory effect of DES in the effector phase of the DTH response, spleen, lymph node, and peritoneal cells from DES-treated animals were adaptively transferred to hosts sensitized with oxazolone 9 days earlier, and the influence on the DTH response measured. The data from this experiment are shown in Figure 3. No evidence of DES-induced suppressor cells in spleen, lymph node, or the peritoneal cavity could be detected. Moreover, no significant effects of transfer of serum from DES-treated animals were found in a similar system.

DISCUSSION

The delayed hypersensitivity response to oxazolone is dependent on specific stimulation of T lymphocytes capable of releasing biologically active mediators, which in turn results in the accumulation of mononuclear cells in the vicinity of the stimulating

TABLE I. Effect of Treatment With Diethylstilbestrol (DES) or Estramustine Phosphate (EMP) on the Delayed-Hypersensitivity Response to Oxazolone in Male B6 Mice

Treatment	n ^c	Effect value ^a Treatment period ^b		Effector phase
		Sensitization phase	n ^c	
Control	6	3.02 ± 0.07	5	3.06 ± 0.15
DES 5.6 mg/kg	4	2.78 ± 0.15	5	2.78 ± 0.12
EMP 30 mg/kg	4	2.58 ± 0.16	4	3.39 ± 0.05

^aEffect value expressed as difference between log cpm experimental ear and log cpm control ear.

^bTreatment during sensitization includes 2 days before as well as 3 days after sensitization. DES was given as a single daily SC injection, EMP 2 daily IP injections. Treatment during the effector phase includes 2 days prior to and the same day as challenge was performed.

^cNumber of animals in each group; n.

TABLE II. Effect of Pretreatment With Cyclophosphamide (Cy) on Diethylstilbestrol (DES) or Estramustine-Phosphate- (EMP-) Induced Inhibition of the Delayed Hypersensitivity Response to Oxazolone in Male NMRI Mice

Pretreatment ^d	Treatment ^c	n ^b	Effect value ^a
			Mean/± SE
None	None	16	2.83 ± 0.06
CY 100 mg/kg	None	16	3.23 ± 0.09
None	DES 5.6 mg/kg	8	2.60 ± 0.07
CY 100 mg/kg	DES 5.6 mg/kg	7	2.73 ± 0.07
None	EMP 100 mg/kg	5	2.22 ± 0.18
CY 100 mg/kg	EMP 100 mg/kg	7	2.58 ± 0.15

^aEffect value expressed as log (cpm experimental ear—cpm control ear).

^bNumber of animals, n.

^cTreatment during sensitization includes 2 days before as well as 3 days after sensitization. DES was given as a single daily SC injection, EMP 2 daily IP injections.

^dSingle-dose CY IP 3 days prior to sensitization.

antigen [22]. Also associated with the cell infiltrate is an increase in vascular permeability, which recent studies have shown to involve an important role of mast cells and basophils [23]. The present paper demonstrates that EMP as well as DES is able to interfere with this process, leading to a decreased influx of mononuclear cells to the area of antigen application in sensitized animals.

The doses of EMP used in this study are equal to or lower than those affecting DNA synthesis in the prostate of the rat [24], indicating that the immune system may be as sensitive as the prostate to EMP. Recent studies from our laboratory indicate that EMP does not interfere with natural killer cell activity during treatment of patients with cancer of the prostate [10], nor was the responsiveness of peripheral blood lymphocytes to T-cell mitogens impaired [25].

Some indications of the mechanism of action of EMP on the DTH response have been revealed through this study. The nitrogen mustard moiety of EMP has alkylating properties and may exert its effect by inhibition of DNA synthesis and cellular proliferation. Induction of a DTH response depends on clonal proliferation of T lymphocytes bearing specific receptors for the inducing antigen. EMP has been found to be a potent

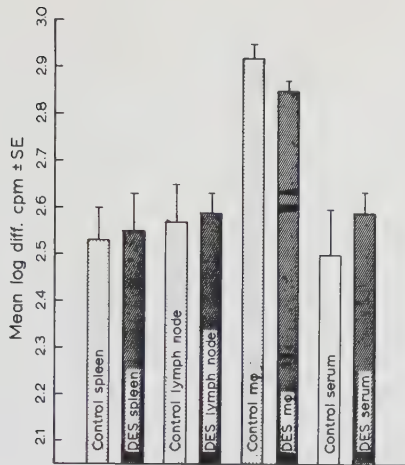


Fig. 3. Effect on delayed hypersensitivity response of cells or serum adaptively transferred from DES-exposed animals to control animals sensitized with oxazolone 9 days earlier. Pretreatment consisted of daily injections with 5.6 mg/kg DES (DES) or olive oil (control) for 7 days.

inhibitor of lymphocyte proliferation *in vitro* [25, 26]. The finding of a strong inhibitory effect of EMP confined to the sensitization phase supports the assumption that EMP acts through inhibition of cellular proliferation.

EMP thereby prevents the induction of cytotoxic T lymphocytes but is not able to interfere with their expression. Recent observations show that EMP has direct cytotoxic effects preferentially on T lymphocytes [26]. A general depletion of T lymphocytes may thus at least partially account for the reduced DTH response after exposure to EMP. However, the lack of effect during the effector phase may indicate that the cytotoxic activity needs some time to become evident or that mechanisms in addition to direct cytotoxicity are important.

EMP has only weak estrogenic properties and metabolic studies indicate that estradiol is only a minor metabolite of EMP *in vivo* [27]. Estrogen-related inhibition of the DTH response is therefore not a very likely consequence of EMP exposure. However, low doses of estrogens may stimulate the immune system [28] and the weak tendency to augmentation of the response after EMP treatment in the effector phase may reflect estrogenic activity.

DES have been reported to have immunosuppressive properties in experimental as well as human systems [8, 9, 11–14], and the present study confirms the results of Luster et al [29] finding a depression of DTH response to tuberculin after treatment with DES. Also, in the latter study the effect of DES was most pronounced when given after sensitization. The dominating effect of DES in the effector phase could be interpreted as a restricted persistence of the immunosuppressive effect of DES. This suggestion is supported by recent observations from our laboratory showing that DES-induced inhibition of mitogen responsiveness is overcome within 2 wk after cessation of treatment [26]. However, a direct effect on memory cell function cannot be readily dismissed.

DES induced thymic necrosis in various mouse strains [30] and acute exposure of adult mice lead to a depletion of T lymphocytes [26, 29]. Moreover, the T-lymphocyte subpopulation mostly affected by DES was of the phenotype Ly 1, the T cell responsible

for induction of the DTH response via the recruitment of Ly 23 effector cells [31]. A simple reduction in the number of T lymphocytes may thus offer an explanation for the reduced DTH response. Estrogens are well-known stimulators of the mononuclear phagocytic system [11, 29] and may also induce nonspecific suppressor cells of the macrophage type [29]. To gain further insight into alternative mechanisms of DES-induced inhibition of DTH response, the possible involvement of various suppressor cells was investigated. However, removal of potential T suppressor lymphocytes by pretreatment of the mice with Cy [20] did not restore the DTH response to control values. In addition, no evidence of suppressor cells either of T-lymphocyte or macrophage type could be detected by an adoptive transfer system, nor were any humoral suppressive factors found.

Estrogens influence the vascular ground substance, rendering it less permeable [32] and may thus interfere with the locomotion of effector cells in the site of antigen application. This effect would also decrease the availability of lymphokines and are consistent with the preferential effect of DES in the effector phase.

In summary, the present study on two commonly used drugs for treatment of prostatic carcinoma show that they are both able to interfere with specific parts of an in vivo immunocompetence assay. Their qualitatively different actions indicate that they act via different mechanisms that further studies are needed to clarify.

ACKNOWLEDGMENTS

This work was supported by The Norwegian Cancer Society (Landsforeningen mot kreft), The Norwegian Council for Science and The Humanities (NAVF), and AB LEO Research Foundation, Helsingborg, Sweden.

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EFFECTS OF DIETHYLSTILBESTROL AND ESTRAMUSTINE PHOSPHATE (ESTRACYT) ON LYMPHOID CELL POPULATIONS AND MITOGEN RESPONSIVENESS IN MALE MICE

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ABSTRACT

Concentrations of diethylstilbestrol phosphate (DES-P) and estramustine phosphate (EMP) above 10^{-5} M in cultures of spleen lymphocytes from adult male mice resulted in a dose related inhibition of both Con A and LPS induced lymphocyte proliferation. Male mice injected with 5.6 mg./kg. DES daily for 7 days had a significantly reduced responsiveness to both Con A and LPS compared to mice injected with olive oil only. Spleen lymphocytes from male mice treated with 100 mg./kg. EMP showed a reduction of Con A induced mitogenesis whereas they exhibited a significantly enhanced response to LPS. The effects of DES and EMP on Con A and LPS induced blastogenesis were abolished within 2 weeks after cessation of treatment.

DES treatment resulted in preferential depletion of splenic and lymph node T lymphocytes and a disproportionate T lymphocyte subpopulation with respect to Ly subclasses. Exposure to 30 or 100 mg./kg. EMP resulted in a dose related loss of mononuclear cells both in spleen and lymph nodes.

T lymphocytes predominantly of the Ly 1 phenotype were most sensitive to EMP. Co-cultures of spleen lymphocytes from normal mice and Mitomycin C blocked spleen cells from either normal or treated mice (DES or EMP) gave no convincing evidence of suppressor cell activity in the population of spleen mononuclear cells.

Estrogen treatment has been shown to alter the immune response in both man and laboratory animals.¹ Exposure of adult mice to diethylstilbestrol (DES), a synthetic, non-steroidal estrogen inhibited the expression of delayed hypersensitivity response, depressed antibody production and seriously impaired host resistance to certain infections and transplantable syngeneic tumor cells.^{2,3}

The effects of acute adult exposure to DES are usually temporary while neonatally DES exposed mice show persistent alterations of both humoral and cellular immunity.⁴ DES-treatment impaired general as well as tumor-specific immunity in patients with cancer of the prostate^{5,6} and substantially reduced natural killer (NK) cell activity in peripheral blood.⁷

Estramustine phosphate (EMP, Estracyt®) is an estrogen-cytostatic complex mainly used in the therapy of hormone resistant prostatic cancer. Although its therapeutic effects are well documented,^{8,9} the mechanisms of action of EMP are not fully understood, but appear to be at least partially distinguishable from mere estrogen effects.¹⁰

In contrast to DES, EMP had no influence on NK activity in peripheral blood from patients treated for cancer of the prostate⁷ and did not alter delayed hypersensitivity reactions.¹¹ The aim of the present study was to evaluate further the effects of exposure to these drugs on the immune system of adult male mice.

MATERIALS AND METHODS

Mice. All mice used were males belonging to a randomly bred NMRI stock kept at our institute, or to the inbred strain C57Bl/6 (B6) purchased from G1. Bomholtgård, Denmark. They were fed a standard diet and given water ad libitum. The NMRI mice were used at 8–12 weeks of age and had a mean weight of 35 ± 5 gm. B6 mice were used at 8–10 weeks of age and had a

mean weight of 25 ± 5 gm. The B6 congenic lines B6 Ly-1.1 and B6 Ly-2.1 used in the specificity controls of Ly antisera were a kind gift from Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York.

Drug treatment. The experimental animals were exposed to DES or EMP daily for 7 days before testing. DES treated mice received 1.4 mg. or 5.6 mg./kg. body weight in 0.05 ml. olive oil as daily subcutaneous injections. EMP were given intraperitoneally twice a day at a daily dose of 15 or 100 mg./kg. body weight. Control animals were given olive oil or saline only. Experimental and control mice were also assayed for mitogen responsiveness 2 weeks after the last drug injection to establish the duration of the drug effects.

Lymphocyte cultures. The mice were killed by cervical dislocation. Their spleens were rapidly removed under sterile conditions, stripped free from adhering tissue, placed in phosphate buffered saline (PBS, pH 7.4) and minced with forceps. The resulting cell suspension was layered onto a Ficoll-Isopaque (Lymphoprep, Nyegaard, Oslo, Norway) gradient solution in centrifuge tubes. Mononuclear cells were separated by the method of Bøyum¹² with the use of centrifugation at $800 \times g$ for 20 minutes. The cells were washed twice in PBS and resuspended to 2×10^6 cells/ml. in RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Bio-Cult, GIBCO, Grand Island, New York) supplemented with 10 per cent heat-inactivated fetal calf serum (FCS, Bio-Cult, GIBCO) and 100 μ g. streptomycin/100 IU penicillin per ml. (complete medium). The viability of the cells was checked when the culture was started and was always greater than 90 per cent with the use of the trypan blue exclusion test.

The lymphocytes were cultured in round-bottom microtiter plates (Greiner Original, Greiner and Co, Cambridge, Massachusetts) at 37°C in 5 per cent CO₂ and 95 per cent air with 98 per cent humidity. Each well contained 2×10^5 cells in 0.2 ml. complete medium. In addition, the cultures were supplied with 2.5, 5 or 10 μ g. concanavalin A/ml. (Con A, Sigma Chemical Co.) or 5, 25 or 100 μ g. lipopolysaccharide/ml. (LPS from E. coli serotype No. 055:B5, phenol-extracted, Sigma Chemical Co.). The maximum stimulatory response was in initial experi-

Accepted for publication March 1, 1982.

Supported by grants from the Norwegian Cancer Society (Landsforingen mot Kreft) and A/B LEO Research Foundation, Helsingborg, Sweden.

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ments always found within these ranges of mitogen concentrations.

From every spleen, triplicate cultures were run at every concentration of Con A or LPS used (unstimulated cultures inclusive).

Forty-eight hours after the cultures were started, 0.5 μ Ci (methyl- 3 H-dThd, sp. act. 5 Ci/mmol., Radiochemical Centre, Amersham, U. K.) was added to each culture well. This amount of radioactivity was in pilot experiments found to be the lowest giving a maximum stimulation index (cpm mitogens stimulated cultures/cpm culture without mitogen) when various concentrations of 3 H-dThd was added to the cultures. The cells were harvested onto glass fiber filters (Glass Microfibre Paper GGS, Whatman LTD, U. K.) 24 hours later with a Skatron Multiple Cell-Culture Harvester (Skatron A/S, Lierbyen, Norway). The filters were dried at 60°C and put into counting vials containing 3 ml. scintillation fluid (fluorochromes in toluene) and radioactivity measured in a Packard Tri-Carb liquid scintillator. The counting efficiency was 40 per cent as determined by internal standard. The results were expressed as cpm. In pilot experiments, a check was made that a linear regression existed between the cpm values and the number of cells in the culture tubes (from 50,000 to 400,000 cells with intervals of 50,000 cells) and between the cpm values and the number of cells added to the glass-fiber filter (from 100,000 to 400,000 cells with intervals of 100,000 cells). In some experiments normal spleen cells were co-cultured with Mitomycin C (MMC, Sigma, St. Louis, Missouri) treated mononuclear spleen cells from either treated or control mice. MMC-treatment was performed by incubation (50 μ g. MMC/ 10^7 cells/ml.) for 40 minutes at 37°C followed by 3 washes in RPMI-1640. Normal spleen lymphocytes in a final number of 2×10^5 per well were co-cultivated with 2×10^5 MMC-blocked spleen cells in 0.2 ml. complete medium with 5.0 μ g. Con A/ml. or 25.0 μ g. LPS/ml. as well as without mitogens.

In vitro effects of diethylstilbestrol phosphate (DES-P, Honvan®) and EMP were investigated in cultures stimulated with optimal doses of mitogen (5 μ g./ml. Con A; 25 μ g./ml. LPS).

Antisera. Monoclonal Anti-Thy 1.2 antiserum was from Olac Ltd., U. K. Ly antisera were a gift from Dr. F. W. Shen, Memorial Sloan-Kettering Cancer Center, New York. For details on the preparation and use of the Ly antisera, see Shen and associates.¹³ Since all experiments were conducted with B6 mice which have the phenotype Ly 1.2, 2.2, 3.2, the following control for Ly specificity was included as recommended by Shen and associates.¹³ Briefly, for each antiserum used it was shown that the putative Ly specific activity of the antiserum was removed by absorption of the antiserum with thymocytes of B6 mice, but not by absorption with thymocytes of B6-Ly congenic mice of the alternative allotype.

Complement-dependent cytotoxicity assay. Cell suspensions of spleen or lymph nodes (axillary and mesenteric lymph nodes) were prepared on ice in PBS supplemented with 10 per cent FCS. Erythrocytes were removed by hypotonic shock treatment. The cell number was adjusted to 2×10^7 cells/ml. in PBS-FCS, and the cells labeled with 100 μ Ci 51 Cr/ml. (sodium 51 Chromate, specific activity 350–600 mCi/mg., The Radiochemical Centre, Amersham, U.K.) by incubation in a water bath at 37°C for 45 minutes with gentle shaking.¹⁴ The labeled cells were washed twice in 50 ml. PBS-FCS and the cell number adjusted to 5×10^6 /ml.

Fifty microliters (2.5×10^5 cells) of the cell suspension were incubated with 50 μ l. antiserum (predetermined optimal dilution in PBS-FCS) or with normal mouse serum (NMS) for 30 minutes on ice. They were washed once and resuspended in 100 μ l. freshly thawed rabbit serum (complement-source, predetermined optimal dilution), before further incubation for 30 minutes at 37°C. A single pool of rabbit serum selected for low natural cytotoxicity and high complement activity was used throughout. After incubation, the cells were spun down, and 50

μ l. of the supernatant was collected and counted in a Searle 1185 Gamma Spectrophotometer.

Percentage lysis was calculated by the formula:

$$\frac{\text{cpm antiserum} - \text{cpm (NMS)}}{\text{cpm (maximum release)} - \text{cpm (NMS)}} \times 100$$

Maximum release was determined by incubation of targets cells with H_2O followed by 3 sequences of freeze-thawing.

Enumeration of Ly-Cell sets. The proportions of Ly 1, Ly 23, and Ly 123 cell sets were estimated from the percentage lysis by Ly 1 and Ly 2 antiserum used alone or in combination as described earlier.¹⁵ Data for Ly sets were expressed as percentage of total Ly positive cells.

Enumeration of B cells. Cells bearing surface immunoglobulin (Slg) were enumerated by direct immunofluorescence. Mononuclear cell suspension was obtained from the spleens as described previously. The cells were preincubated at 37°C for 1 hour with 3 washings to eliminate cytophilic antibodies.¹⁶ TRITC-labeled polyvalent rabbit antibodies to mouse immunoglobulins were obtained from Cappel Laboratories (Cochranville, Pennsylvania, lot 10225). The antiserum was ultracentrifuged immediately prior to use to remove aggregates. Preincubation with a mouse immunoglobulin preparation completely abolished the fluorescence.

Fifty microliters of cell suspension (10^7 /ml.) in PBS supplemented with 10 per cent bovine serum albumin (BSA, Sigma) were incubated with predetermined optimal dilution of conjugated antiserum for 45 minutes at 4°C. After 3 washings in PBS-BSA the cells were mounted and examined in a Zeiss microscope equipped for epifluorescence. Duplicate samples of at least 200 cells were counted.

Phagocytic cells were visualized after ingestion of latex particles.¹⁷

Statistical analysis. All experiments were performed in at least 3 separate upsets. The results were compared using Variance analysis or Student's *t* test. Before doing these comparisons we made sure that error variance of the groups involved did not differ significantly.

RESULTS

DES

In vitro effects of DES-P mitogen response of spleen lymphocytes from male NMRI mice. The effects of various concentrations of DES-P on Con A or LPS stimulation of spleen lymphocytes are shown in figure 1. DES-P from 10^{-5} to 10^{-3} M

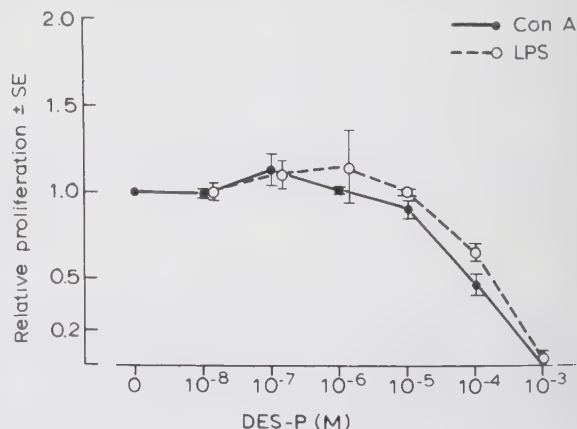


Fig. 1. Con A and LPS stimulated proliferation of normal spleen lymphocytes from 3 10-week-old male NMRI mice in the presence of various concentrations of DES-P (Honvan). Proliferation expressed relative to control values.

in the cultures resulted in a dose related inhibition of mitogenesis, whereas 10^{-7} M DES-P augmented the Con A and LPS response to some extent.

Mitogen response of spleen lymphocytes from DES treated male NMRI mice. The results are presented in figures 2 and 3. A treatment regimen of 5.6 mg./kg. DES daily reduced the uptake of ^3H -dThd significantly in spleen lymphocyte cultures stimulated with Con A compared to controls ($p < 0.001$). No significant alterations were observed in lymphocyte cultures from mice treated with 1.4 mg./kg. DES daily. LPS stimulated cultures were inhibited by the highest dose of DES ($p < 0.001$) whereas 1.4 mg. stimulated the response significantly ($p < 0.05$).

Mitogen response of spleen lymphocytes from male NMRI mice 2 weeks after cessation of DES treatment. In order to evaluate the duration of effects of DES exposure on Con A and

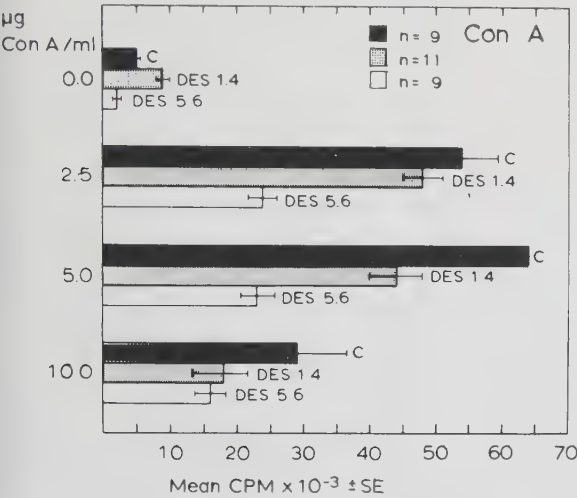


FIG. 2. Mitogen response to Con A of spleen lymphocytes from 8 to 12-week-old male NMRI mice given s.c. injections of olive oil (C, controls) or 1.4 or 5.6 mg./kg. DES daily for 7 days. n = no. of animals in each group.

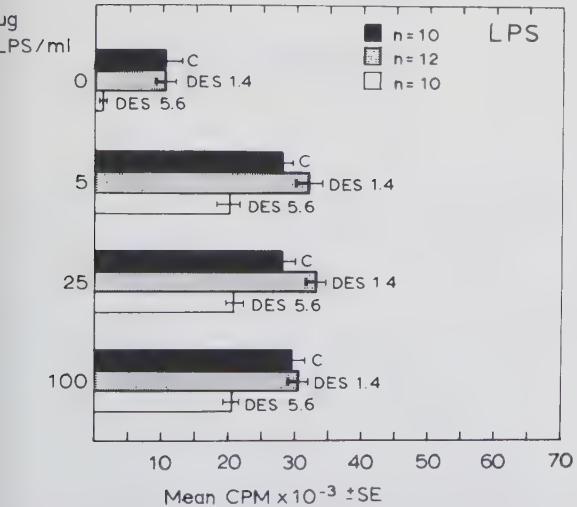


FIG. 3. Mitogen response to LPS in cultures of spleen lymphocytes from 8 to 12-week-old male NMRI mice given s.c. injections of olive oil (C, controls) or 1.4 or 5.6 mg./kg. DES daily for 7 days. n = no. of animals in each group.

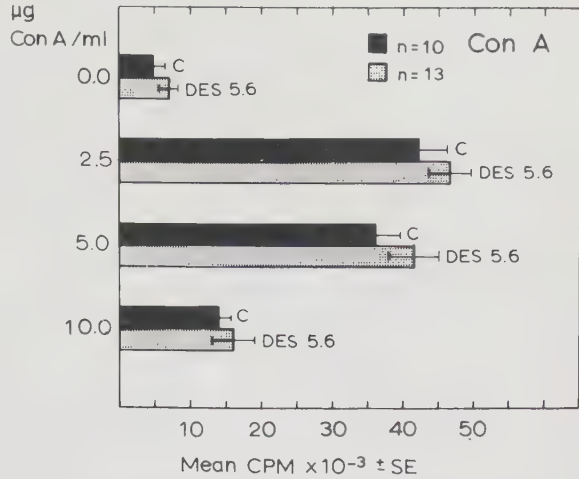


FIG. 4. Mitogen response to Con A of spleen lymphocytes from 8 to 12-week-old male NMRI mice given s.c. injections of olive oil (C, controls) or 5.6 mg./kg. DES daily s.c. for 7 days and assayed 2 weeks after the last injection. n = no. of animals in each group.

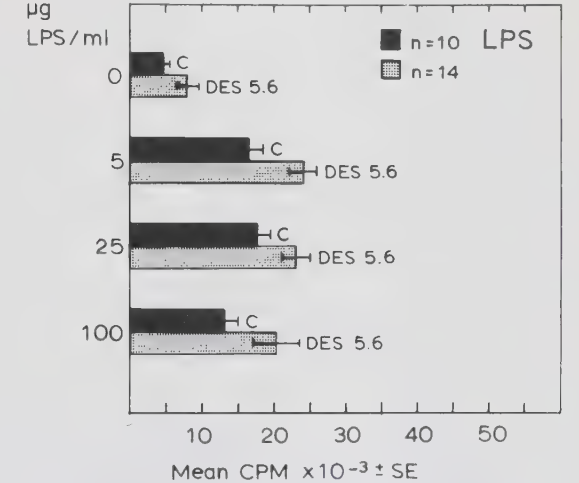


FIG. 5. Mitogen response to LPS of spleen lymphocytes from 8 to 12-week-old male NMRI mice given s.c. injections of olive oil (C, controls) or 5.6 mg./kg. DES daily for 7 days and assayed 2 weeks after the last injection. n = no. of animals in each group.

LPS response of spleen lymphocytes, lymphocytes from control or experimental mice were examined 2 weeks after the last injection. The results from these experiments are presented in figures 4 and 5. The magnitude of response to Con A and LPS was comparable to control response except that lymphocytes from DES treated mice cultures in presence of 5 µg. LPS/ml. resulted in significant enhancement of the response ($p < 0.05$) (fig. 5).

Mitogen response in co-cultures of lymphocytes from normal and DES treated male B6 mice. Pooled spleen lymphocytes from control or DES (5.6 mg./kg.) treated mice were cultured separately or co-cultured with MMC blocked spleen cells from normal or DES exposed animals. Exposure to DES of male B6 mice resulted in inhibition of proliferation of spleen lymphocytes stimulated with Con A or LPS compared to the response of control lymphocytes. The per cent inhibition of Con A response due to DES treatment was 44 per cent of control

TABLE 1. Mitogen response of spleen lymphocytes co-cultured with MMC blocked spleen cells from control or DES treated male B6 mice

Responder* Cells	Co-cultured† Cells	³ H dThd Incorporation (cpm)‡		
		Unstimulated	Con A	LPS
Normal	None	1.942 ± 865	16.844 ± 4.302	14.639 ± 1.410
DES	None	1.528 ± 580	9.497 ± 3.739 (44% ↓)	10.224 ± 1.727 (30% ↓)
Normal	Normal, MMC-blocked	1.773 ± 613	19.159 ± 7.474	9.538 ± 1.517
Normal	DES, MMC-blocked	1.695 ± 590	16.023 ± 6.947 (16% ↓)	9.348 ± 2.133 (2% ↓)

* Spleen lymphocytes from control or DES treated mice (5.6 mg./kg. daily).

† Mitomycin C treated spleen cells from control or DES exposed mice.

‡ Mean cpm ± SE of 3 experiments. Each experiments represents pooled spleen cells from at least 4 mice

TABLE 2. Effect of treatment with DES or EMP on mononuclear cell population in spleen and lymph nodes of male B6 mice.

	Per Cent of Mononuclear Cells							
	Spleen				Lymph Nodes			
	C	DES	EMP (100 mg./kg.)	EMP (30 mg./kg.)	C	DES	EMP (100 mg./kg.)	EMP (30 mg./kg.)
Thy 1.2	42	28	11	21	75	66	29	38
Slg	45	50	76	55	15	18	48	40
Latex ingestion	10	14	6	10	12	12	9	15
Ly, total	39	25	11	20	69	64	26	37
Ly 123	32	37	45	39	18	23	37	30
Ly 1	54	40	38	41	60	50	48	49
Ly 23	14	23	17	20	21	27	15	21
Mononuclear cells (× 10 ⁶ per spleen or mouse (LN))	19.0	19.8	4.1	9.4	3.8	3.4	0.46	0.54

response and the corresponding per cent inhibition of LPS blastogenesis 30 per cent.

Proliferation of spleen lymphocytes in co-cultures of normal and MMC blocked spleen mononuclear cells stimulated with Con A or LPS were reduced 16 and 2 per cent compared to proliferation of appropriate control cultures.

Effects of DES treatment on lymphoid cell populations. The total number of mononuclear cells recoverable in suspension per spleen or pooled lymph nodes from DES treated B6 mice (5.6 mg./kg.) were comparable to the numbers found in control animals (table 1). DES treatment reduced the total number of T cells per spleen about 30 per cent and resulted in a disproportionate T cell population. The Ly 1 subpopulation appeared to be most sensitive to DES exposure and was reduced to about 50 per cent of normal values.

About 20 per cent of the T cells in lymph nodes were lost after DES exposure. Again DES treatment affected mostly Ly 1 cells. As a consequence of the depleting effect of DES treatment on spleen T cells, the B cell population and to some extent macrophages were enriched. The number of B cells and macrophages in lymph nodes from DES exposed mice appeared to be unaltered compared to controls (table 2).

EMP

In vitro effects of EMP on mitogen response of spleen lymphocytes from male NMRI mice. As shown in figure 6, low concentrations (10⁻⁶ to 10⁻⁸ M) of EMP in vitro stimulated Con A induced mitogenesis, whereas concentrations greater than 10⁻⁴ M were strongly inhibitory to both Con A and LPS induced lymphocyte proliferation.

Mitogen response of spleen lymphocytes from EMP treated male NMRI mice. In figure 7 the effects of treatment with different doses of EMP on responsiveness to Con A and LPS are shown. The Con A response of spleen lymphocytes from experimental animals showed a dose related inhibitory effect of EMP compared to that of controls. One hundred milligrams per kilogram of EMP daily significantly reduced Con A induced lymphocyte proliferation ($p < 0.001$), whereas 15 mg./kg. gave a small but not significant reduction.

In contrast to the inhibitory effect of EMP exposure on Con A induced lymphoproliferation, exposure to the highest dosage

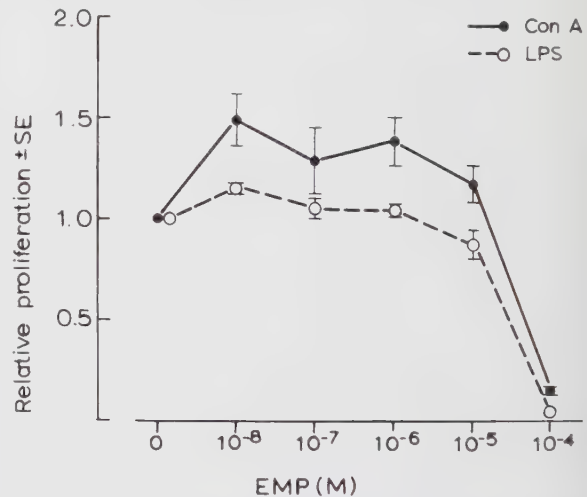


FIG. 6. Con A and LPS stimulated proliferation of normal spleen lymphocytes from 3 10-week-old male NMRI mice in the presence of various concentrations of EMP (Estracyl). Proliferation expressed relative to control values.

of EMP significantly augmented the LPS response of spleen lymphocytes ($p < 0.005$) (figure 8). The lower dose of EMP did not alter LPS induced lymphocyte proliferation significantly.

Mitogen response of spleen lymphocytes from male NMRI mice 2 weeks after cessation of EMP treatment. The effect of exposure to the highest dose of EMP appeared to be short-lived. As shown in figure 9, the suppressive effect of EMP exposure on Con A response of spleen lymphocytes was abolished within 2 weeks after the last injection of EMP. The LPS responsiveness of spleen lymphocytes from EMP exposed mice assayed 2 weeks after cessation of the treatment was comparable to that of control animals (figure 10).

Mitogen response in co-cultures of lymphocytes from normal and EMP treated male B6 mice. The results are presented in

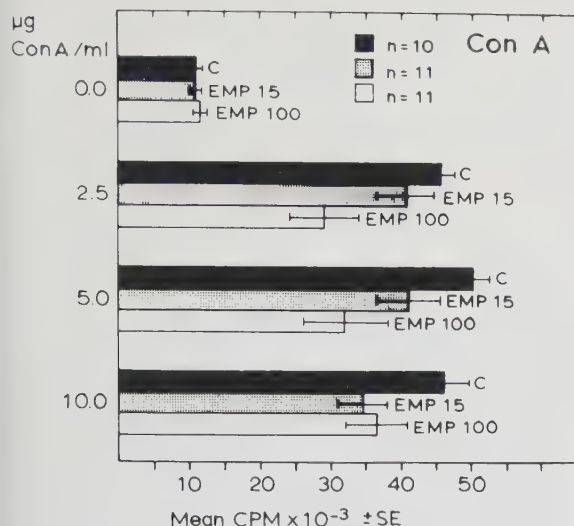


FIG. 7. Mitogen response to Con A of spleen lymphocytes from 8 to 12-week-old male NMRI mice given i.p. injections of saline (C, controls) or 15 or 100 mg./kg. EMP (Estracyt) for 7 days. n = no. of animals in each group.

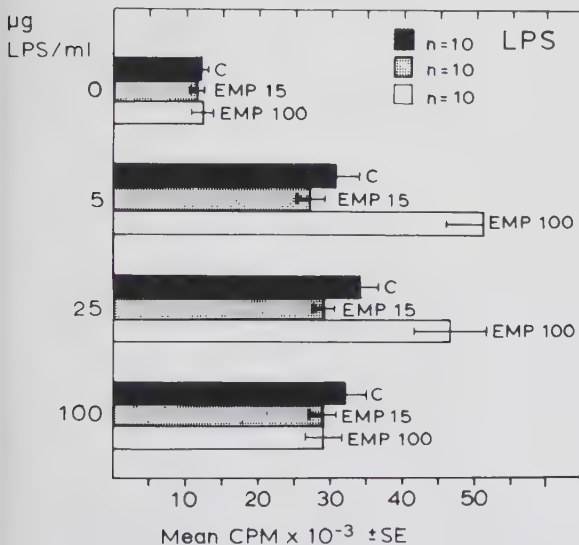


FIG. 8. Mitogen response to LPS of spleen lymphocytes from 8 to 12-week-old male NMRI mice given i.p. injections of saline (C, controls) or 15 or 100 mg./kg. EMP (Estracyt) for 7 days. n = no. of animals in each group.

table 3. Exposure to EMP (30 mg./kg.) reduced the lymphocytes response to Con A 27 percent, whereas LPS stimulated proliferation was augmented 35 per cent to that of control level. Correcting for dose difference, the effects of EMP treatment in B6 mice was comparable to the effects of EMP exposure in NMRI mice. Co-cultivation of spleen lymphocytes from control animals with MMC-blocked spleen mononuclear cells from EMP treated mice reduced the response to Con A and LPS 17 and 21 per cent, respectively.

Effects of EMP treatment on lymphoid cell populations. EMP exposure in B6 mice resulted in a substantial loss of mononuclear cells from spleen and lymph nodes. Spleen and

lymph nodes from EMP exposed mice were preferentially depleted of T cells in a dose related fashion (table 3). Ninety-four and 75 per cent of splenic T cells were eliminated from mice exposed to 30 or 100 mg./kg. EMP daily. The total number of splenic B cells and macrophages was reduced to a much lesser extent than the number of T cells and as a consequence they were relatively enriched. As for DES treatment the subclass of T cells bearing the Ly 1 marker appeared to be most sensitive to EMP treatment in mice resulting in a disproportionate T lymphocyte populations.

DISCUSSION

Mitogens are polyclonal activators of lymphocytes and have been widely used for characterization of lymphocyte subpopulations and as a measure of general immunocompetence.¹⁸ The

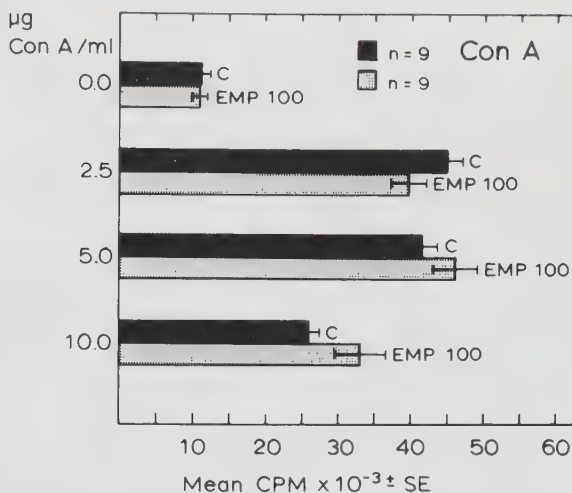


FIG. 9. Mitogen response to Con A of spleen lymphocytes from 8 to 12-week-old male NMRI mice given i.p. injections of saline (C, controls) or 100 mg./kg. EMP (Estracyt) 7 days and assayed 2 weeks after the last injection. n = no. of animals in each group.

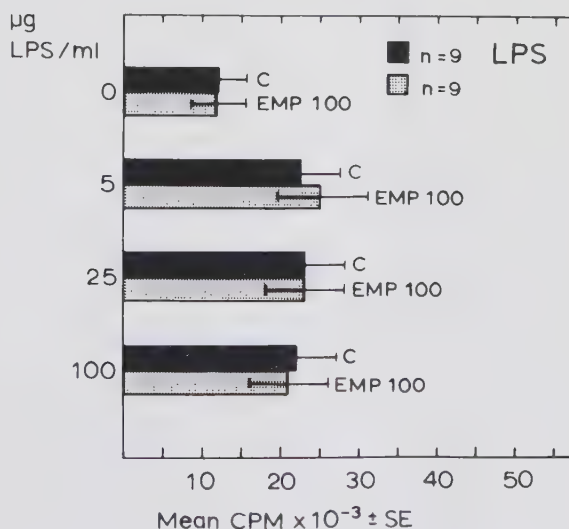


FIG. 10. Mitogen response to LPS of spleen lymphocytes from 8 to 12-week-old male NMRI mice given i.p. injections of saline (C, controls) or 100 mg./kg. EMP (Estracyt) for 7 days and assayed 2 weeks after the last injection. n = no. of animals in each group.

TABLE 3. Mitogen response of spleen lymphocytes co-cultured with MMC blocked spleen cells from control or EMP treated male B6 mice

Responder* Cells	Co-cultured† Cells	H dThd Incorporation (cpm)‡		
		Unstimulated	Mitogenstimulated	
			Con A	LPS
Normal	None	922 ± 374	28,321 ± 3,856	7,528 ± 2,050
EMP	None	1,099 ± 331	20,651 ± 1,474 (27% ↓)	10,132 ± 2,527 (35% ↑)
Normal	Normal, MMC-blocked	939 ± 341	30,560 ± 1,933	6,805 ± 2,476
Normal	EMP, MMC-blocked	805 ± 309	25,513 ± 2,226 (17% ↓)	5,360 ± 2,434 (21% ↓)

* Spleen lymphocytes from control or EMP treated mice (30 mg./kg. daily).

† Mitomycin C treated spleen cells from control or EMP exposed mice.

‡ Mean cpm ± SE of 4 experiments. Each experiment represents pooled spleen cells from at least 4 mice.

present study demonstrates that DES is a potent inhibitor of mitogen induced proliferation of mouse spleen lymphocytes in vitro at concentrations equal to or higher than 10^{-5} M. The results are in line with the findings of Ablin and associates¹⁹ and Harty and associates²⁰ showing that estrogens as well as steroid hormones in general inhibit proliferation of lymphocytes at pharmacologic concentrations. Mouse lymphocytes appear to be more susceptible to inhibition of DES in vitro than human peripheral blood lymphocytes as only about 50 per cent inhibition of human peripheral blood lymphocytes to T cell mitogens were achieved even at the highest concentrations of DES.¹⁹

The mechanism behind the effects of DES in vitro on blastogenesis is obscure. Estrogen receptors have not been detected either in resting or lectin stimulated lymphocytes and estrogens do not cross-react with glucocorticoid receptors present in lymphocytes and thymocytes.²² Moreover, DES exhibits blocking effect on mitogen response only at pharmacologic concentrations indicating a non-specific, nonreceptor-mediated mechanism. Accumulating evidence indicate that estrogens have important effects on the cell membrane.²³ In target cells estrogens may increase adenylate cyclase activity²⁴ and alter the calcium flux through the cell membrane.²⁵ The possibility also exists that DES reacts with serum factors necessary for transition of lymphocytes in culture from G1 to S-phase of the cell cycle.²⁶ Instead of acting directly on mitogenresponsive lymphocytes, DES may interfere with subpopulations of spleen mononuclear cells delivering signals regulating proliferation.

The present study documents substantial depressive effects of 5.6 mg./kg. DES daily on proliferation of lymphocytes stimulated with T- or B-cell mitogens. The suppressive effect of the highest dose of DES exposure in mice on lymphocyte reactivity appears to be short-lived as the ability to respond to mitogens is restored 2 weeks after cessation of treatment. The enhancement of mitogen response seen after 2 weeks could represent some kind of compensatory phenomena.²⁷ Luster and associates found DES-induced suppressor activity in the spleen macrophage population.² The present study does not provide convincing evidence supporting the concept of suppressor macrophages induced by DES. There are several factors which could account for this somewhat conflicting results concerning induction of suppressor macrophages such as sex effects (females versus males), strain differences or treatment regimen.

Elimination of T cells belonging to the Ly 1 phenotype may at least in part be responsible for the suppression of the proliferative response to the T cell mitogen. T lymphocytes play an important role in the regulation of various immune responses.²⁸ In the mouse, T cells bearing the Ly 1 phenotype appear to be inducer cells of delayed type hypersensitivity response, act as helper cells in antibody production and amplify the function of cytotoxic and suppressor T cells.^{29,30} T cells of the phenotype Ly 23 generally act as suppressor cells whereas Ly 123 cells have been implicated as intermediary cells and amplifier cells in various immune reactions.³¹ Preferential elimination of Ly 1 cells could thus perturb the regulatory balance between T lymphocyte subclasses in favor of suppression.

In contrast to a dose dependent inhibitory effect of DES exposure on Con A induced lymphocyte proliferation, the high-

est dose of DES suppressed, whereas the lower dose stimulated the LPS response. Stimulatory effects of DES treatment on the mononuclear phagocytic system have been reported in experimental animals.³² Low doses of estrogens have been shown to stimulate, while higher dose inhibit cellular as well as humoral immunity.³³ In the present study DES increased the number of B lymphocytes per spleen. This study provide no evidence indicating a role of suppressor cells operating in LPS stimulated lymphocyte cultures derived from spleens of DES exposed animals. Thus, DES exposure seems to impair the LPS responsiveness of B cells without reducing their number or acting via suppressor cells.

The effects of acute exposure to DES in adult male mice are transient in contrast to the long-term effects of neonatal exposure to DES on the immune system reported by Kalland, who observed a reduced and disproportionate T cell population after neonatally DES exposure.^{4-6,18} The present results concerning the effects of adult exposure to DES in mice are qualitatively similar to these observations.

The results presented in this paper demonstrate that EMP produces a marked inhibition of Con A and LPS induced proliferation of mouse spleen lymphocytes in vitro at concentrations higher than 10^{-5} . The facilitating effect of EMP on Con A induced blastogenesis at concentrations lower than 10^{-5} M was also to some degree observed in a study dealing with in vitro effects of EMP on mitogen response of human peripheral blood lymphocytes (unpublished observations). The mechanisms behind these effects of EMP cannot be fully understood until the metabolism of EMP in spleen cell cultures is revealed, although it seems reasonable to suggest that the effects may be attributed to the alkylating moiety of EMP inhibiting DNA synthesis and cellular proliferation.

The present study demonstrates that exposure to various doses of EMP in adult mice results in a profound elimination of splenic T cells in a dose dependent fashion. B cells and macrophages appear to be less prone to the effects of EMP. The marked reduction of the number of T lymphocytes per spleen after exposure to EMP appear to be responsible for the decreased proliferation of lymphocytes stimulated with T cell mitogen. As a consequence of T cell elimination, there was an enrichment of B lymphocytes in the cultures resulting in increased proliferation to LPS. On the other hand, results from co-culture experiments could indicate that EMP exposure induces suppressor cells contributing to the inhibition of the Con A response. EMP exposure in male mice during the afferent phase of the DTH response reduced the ability to DTH expression without having any effects in the efferent phase.³⁴ This observation again points to an alkylating effect of EMP being its major mechanism as discussed above. The DTH response is initiated by Ly 1 inducer T cells and this cell type is a major contributor to Con A induced lymphocyte proliferation. Thus, the effects of EMP on DTH response and mitogen stimulation are in full agreement.

In contrast to the immunosuppressive effects of EMP exposure in male mice, EMP therapy of patients with prostatic cancer showed no consistent depression of proliferation of peripheral blood lymphocytes to T cell mitogens.²¹ NK activity in

peripheral blood of patients with prostatic cancer subjected to 4 weeks EMP therapy also remained unaffected.⁷ These discrepancies reinforce the necessity of being cautious when comparing the results from animal studies with the human therapeutic situation. Species differences with regard to drug metabolism, dose response relations and routes of drug administration make the situation unclear.

EMP is usually given by mouth or injected intravenously to patients with prostatic cancer, and the appropriate dose is 10–15 mg./kg. daily. However, to reduce the ³H-dThd incorporation of the rat ventral prostate a daily dose of 100 mg./kg. was necessary.⁴⁷ EMP has antigonadotropic effect similar or even higher than estrogens.⁴⁸ The dominating metabolite of EMP in the circulation of man and rat appears to be estramustine, a dephosphorylated metabolite of EMP, whereas estradiol derived from EMP do not play any significant role.⁴⁷ In patients on EMP therapy elevated levels of plasma transcortin and cortisol have been demonstrated.³⁸ On the basis of the present results and knowledge of EMP metabolism in man and rodents it seems reasonable to suggest that the immunosuppressive effects of EMP is not merely estrogen effects since the potent estrogen DES has qualitatively different and less pronounced effects on the immune system. However, it should not be excluded that some of the effects of EMP *in vivo* are mediated by alterations in plasma proteins or hormonal levels.

In summary, the present study on 2 commonly used drugs for treatment of prostatic carcinoma show that they both are able to interfere with mouse lymphocyte function *in vitro* and *in vivo*. Their effects seems to be dependent on the ability to preferentially eliminate T lymphocytes and no convincing evidence of suppressor cell activity was detected.

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Suppression of Antibody Response of Male Mice Exposed to Diethylstilbestrol or Estramustine Phosphate (Estracyt^R)

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The effects of treatment of male mice with diethylstilbestrol (DES) or estramustine phosphate (EMP) on the primary antibody response to sheep red blood cells (SRBC) and lipopolysaccharide (LPS) were investigated using a hemolytic plaque assay for enumeration of antibody-producing cells. DES treatment at a daily dose of 1.4 or 5.6 mg/kg from 2 days prior to and up to 3 days after immunization significantly reduced the number of anti-SRBC as well as anti-LPS-producing cells per 10^6 spleen cells. The 100 mg/kg EMP given as daily intraperitoneal injections significantly diminished the antibody response to both SRBC and LPS. No alteration in the number of anti-SRBC-producing cells per 10^6 spleen cells was detected in spleens from mice receiving 30 mg/kg/day EMP, while the number of anti-LPS-producing cells was reduced.

The immunoregulatory effects of EMP are apparently not a pure estrogen effect, since treatment with doses of estradiol-17 β equivalent to the amounts of estradiol linked to the cytotoxic moiety in 100 mg/kg/day of EMP were without effect. EMP-induced functional impairment in the T-cell population is inferred from the reduced ability of adoptively transferred T cells from EMP-exposed animals to support an antibody response to SRBC in syngeneic nude mice.

Key words: diethylstilbestrol, estramustine phosphate, antibody-secreting cells

INTRODUCTION

Diethylstilbestrol (DES) is a synthetic, nonsteroidal estrogen widely used in the treatment of patients with advanced cancer of the prostate. Several reports have documented that DES has immunosuppressive properties in man as well as in experimental animals [1-5]. However, concern has been expressed as to whether or not DES treatment might add further to the impairment of the immunocompetence observed in patients with advanced cancer of the prostate because of its immunosuppressive effects [4, 6-8]. In the last decade estramustine phosphate (EMP, Estracyt^R), a therapeutic agent effective in controlling advanced prostatic cancer, has been available. EMP is a single molecule combining estradiol-17 β and an alkylating cytostatic compound, nor-nitrogen mustard. The clinical effect of EMP against metastatic and progressive cancer of the prostate is well documented [9].

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In previously reported studies, we compared the immunopharmacological effects of a conventional estrogen regimen and treatment with EMP in patients with cancer of the prostate. EMP treatment did not impair natural killer (NK) cell activity or mitogen responsiveness during a 4-week treatment period, while DES reduced NK-cell activity after 1 week of treatment [3,10]. Moreover, diethylstilbestrol phosphate (DES-P) inhibited proliferation of peripheral blood lymphocytes to the T-cell mitogen phytohemagglutinin [3].

An experimental mouse system was selected to gain further insight into the mechanism behind the immunoregulatory effects of DES and EMP treatment. Our earlier studies showed that DES as well as EMP were able to impair the ability to mount a delayed hypersensitivity response [11]. Both drugs inhibited response to a T-cell mitogen, and DES also suppressed proliferation of spleen lymphocytes stimulated with a B-cell mitogen [12]. EMP appeared to be cytotoxic to mouse T lymphocytes, while DES preferentially eliminated splenic T lymphocytes of the subclass Ly 1 [12].

The present investigation was undertaken to delineate and compare the effect of DES and EMP treatment on the humoral immune response.

MATERIALS AND METHODS

Mice

Male NMRI mice aged 8–12 weeks were obtained from Anticimex (Stockholm, Sweden) or from our own breeding colony. Male C57Bl/6 (B6) nu/+ and nu/nu, aged 8 weeks (purchased from Gl. Bomholtgård, Denmark), were used in the adoptive transfer experiments. All animals were fed a standard pellet diet and given water ad libitum.

Drug Treatment

DES (Sigma Chemicals, London) was dissolved in a minimum of ethanol and diluted with olive oil to appropriate concentrations. Estradiol-17 β (Sigma Chemicals) was suspended in 4% dimethylsulfoxide (DMSO, E. Merck, Darmstadt, West Germany) in phosphate-buffered saline (PBS, pH 7.4). EMP (AB Leo, Helsingborg, Sweden) was dissolved in sterile distilled water. The experimental animals were exposed to DES, EMP, or estradiol-17 β during the induction of the antibody response including 2 days prior to and 3 days after the immunization. DES-treated mice received either 1.4 or 5.6 mg/kg/day in 0.05 ml vehicle as single subcutaneous (sc) injection. EMP was injected intraperitoneally (ip) twice a day at a daily dose of 30 and 100 mg/kg, respectively. In some experiments mice were given ip injections of 48 mg/kg/day estradiol-17 β . This dose was selected because 48 mg estradiol-17 β was equivalent to the amount of estradiol linked to the nor-nitrogen mustard in 100 mg EMP. Control animals were given the different vehicles only according to identical schedules.

In order to determine the effect of EMP treatment on helper T-cell function, 10⁷ enriched spleen cells (see below) from B6 nu/+ males treated for 5 days with 100 mg/kg/day EMP were transferred to B6 nu/nu males. Two days after cell transfer, the mice were primed with sheep red blood cells (SRBC), and the antibody response was recorded as described below.

Assay for Antibody Response

The immunization procedure consisted of ip injections of 0.3 ml 10% (v/v) SRBC in PBS (Statens Bakteriologiska Laboratorium, Stockholm; cells from the same

sheep used throughout these studies) or 10 μ g lipopolysaccharide (LPS, phenol extracted from *Escherichia coli* 055:B5, Sigma Chemicals). On day 5 after immunization, the mice were killed by cervical dislocation. The spleen was removed under sterile conditions, minced with forceps in PBS, and teased through a mesh steel screen. Red cells were removed from the spleen cell suspension by hypotonic shock treatment. After washing three times in PBS, the spleen cells were resuspended in RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (GIBCO, Bio-Cult, Grand Island, NY), and the cell number was adjusted to 2×10^6 /ml RPMI.

In the adoptive transfer experiments, single spleen cell suspensions from EMP-treated or untreated B6 mice were obtained as described above and further enriched for T lymphocytes. Ten milliliters of 10^7 spleen cells/ml were suspended in 20% fetal calf serum (FCS, GIBCO, Bio-Cult) in RPMI and incubated in tissue culture flasks at 37°C for 45 min. Nonadherent cells were aspirated, washed in 10% FCS-RPMI, and adjusted to 10^7 cells/ml. From 2 to 3 ml of the cell suspension was "panned" in plastic petri dishes precoated with anti-mouse IgG-F(ab)₂ fragments, essentially as described by Wsocki and Sato [13]. The nonadherent cells recovered from the dishes consisted of more than 90% T cells as judged by direct immunofluorescence with FITC-labeled monoclonal antibody to Thy 1 antigen (New England Nuclear, Boston, MA). T-cell-enriched spleen cells were washed and resuspended in PBS at 5×10^7 cells/ml. The viability of cells was always checked before use in assay or injected iv and was found to be $\geq 95\%$.

The Cunningham modification of Jernes hemolytic plaque assay for enumeration of antibody-forming cells was used [14]. To detect the number of spleen cells forming antibody to LPS, SRBC were sensitized before use in the assay by the method of Möller and Michael [15]. SRBC or LPS-sensitized SRBC were washed three times in PBS and resuspended in RPMI at 10% (v/v) concentration. Guinea pig serum (GPC, Statens Bakteriologiska Laboratorium, Stockholm, Sweden) was used as complement source in a final concentration 1:15 (predetermined optimal dilution, the same batch was used throughout these studies). The assay was set up in duplicate. All handling of cells took place at room temperature, unless otherwise stated. The chambers were incubated for 45 min at 37°C in 5% CO₂ atmosphere. Direct plaque-forming cells (PFC) to SRBC or LPS were enumerated by counting in inverted light microscope. The number of PFC to unsensitized SRBC was subtracted from the number of LPS-sensitized SRBC to calculate the number of specific anti-LPS-forming spleen cells. In initial studies optimal response to SRBC and LPS was found at day 5 after immunization, and all experiments were subsequently done at this point of time.

Statistical Analysis

All experiments were performed in at least three separate upsets. The results were analyzed using the Mann-Whitney test. The significance levels used are as follows: $0.05 > P \geq 0.01^*$; $0.01 > P \geq 0.001^{**}$.

RESULTS

The effects of EMP treatment of adult male NMRI mice on the primary antibody response to SRBC and LPS are shown in Tables I and II. The 100 mg/kg/day EMP significantly diminished the number of anti-SRBC PFC per 10^6 spleen cells or per spleen ($P > 0.01$). The lower dose of EMP had no significant effect on the antibody response to SRBC. Neither 100 nor 30 mg/kg/day EMP did alter the number of anti-LPS PFC per 10^6 spleen cells, while the calculated number of LPS-specific PFC per

TABLE I. Effect of EMP on the Primary Antibody Response to SRBC^a

Treatment ^b	N ^c	Anti-SRBC PFC	
		Per 10 ⁶ spleen cells (Mean $\pm$ SEM $\times 10^{-3}$)	Per spleen (Mean $\pm$ SEM $\times 10^{-4}$)
Saline	13	5.07 $\pm$ 0.55	23.37 $\pm$ 3.12
EMP 30	16	4.31 $\pm$ 0.53 ^{NS}	17.32 $\pm$ 2.64 ^{NS}
EMP 100	15	2.30 $\pm$ 0.25**	8.36 $\pm$ 0.99**

^aMale NMRI mice immunized ip with 0.3 ml 10% (v/v) SRBC 5 days before assay.

^bTreatment with 30 or 100 mg/kg/day EMP ip from 2 days before immunization and 3 days thereafter.

^cNumber of mice in each group.

**0.01 > P $\geq$ 0.001.

^{NS}Not significant.

TABLE II. Effect of EMP on the Primary Antibody Response to LPS^a

Treatment ^b	N ^c	Anti-LPS PFC	
		Per 10 ⁶ spleen cells (Mean $\pm$ SEM $\times 10^{-2}$)	Per spleen (Mean $\pm$ SEM $\times 10^{-3}$)
Saline	14	4.41 $\pm$ 0.55	14.28 $\pm$ 3.00
EMP 30	11	3.30 $\pm$ 0.49 ^{NS}	7.50 $\pm$ 1.34*
EMP 100	12	4.30 $\pm$ 0.80 ^{NS}	7.70 $\pm$ 1.67*

^aMale NMRI mice immunized ip with 10 μ g LPS 5 days prior to assay.

^bTreatment with 30 or 100 mg/kg/day EMP ip from 2 days before immunization and 3 days thereafter.

^cNumber of mice in each group.

*0.5 > P $\geq$ 0.01.

^{NS}Not significant.

spleen was significantly reduced ($P < 0.05$). In initial studies we found that exposure to 100 mg/kg/day EMP for 5 days before immunization with SRBC also reduced the number of anti-SRBC PFC per 10⁶ spleen cells in male B6 mice (Fig. 1). Adoptive transfer of 10⁷ T-cell-enriched spleen cells from EMP treated nu/+ B6 mice to the nu/nu B6 mice resulted in a significantly diminished antibody response to SRBC compared to the response encountered in nude mice transferred with the same number of normal T-cells (Fig. 2). There was no significant difference of antibody response evoked by SRBC in male NMRI mice treated with 48 mg/kg/day estradiol-17 β compared to the response elicited in vehicle-injected mice (Table III).

Exposure to 1.4 and 5.6 mg/kg/day DES, respectively, resulted in a significant reduction of anti-SRBC as well as anti-LPS PFC per 10⁶ spleen cells or per spleen, except that the lower dose of DES had no significant effect on the number of anti-LPS PFC on a per spleen base (Tables III and IV).

DISCUSSION

The present results indicate that EMP treatment of male mice interferes with the generation of antibody-producing cells to T-dependent as well as T-independent antigens. T lymphocytes belonging to the Lyt 1⁺ 23⁻ subset are essential for helper activity and also responsible for induction of delayed hypersensitivity response [16,17]. Clonal expansion of antigen-specific B cells are dependent on signals generated by

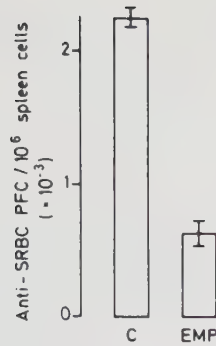


Fig. 1. The number of plaque-forming cells per 10^6 spleen cells of B6 mice immunized with 0.3 ml 10% (v/v) SRBC after exposing the mice to 100 mg/kg/day EMP ip for 5 days (EMP) or saline (C). Each bar represents the mean $\pm$ standard error of the mean plaque-forming cells per 10^6 spleen cells of four mice assayed individually.

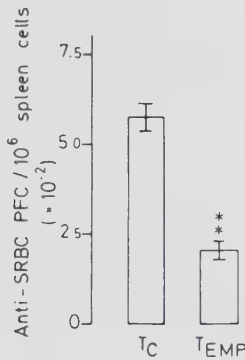


Fig. 2. Anti-SRBC plaque-forming cells of nu/nu B6 mice adoptively transferred with 10^7 T cells from EMP (T_{EMP}-, 100 mg/kg/day for 5 days) or saline (T_C)-treated nu/+ B6 mice. Each bar represents the mean $\pm$ standard error of the mean of plaque-forming cells per 10^6 spleen cells of 10 mice assayed individually. **P < 0.01.

TABLE III. Effect of Estrogen on the Primary Antibody Response to SRBC^a

Treatment ^b	N ^c	Anti-SRBC PFC	
		Per 10^6 spleen cells (Mean $\pm$ SEM $\times 10^{-3}$)	Per spleen (Mean $\pm$ SEM $\times 10^{-4}$)
Olive oil	9	3.31 $\pm$ 0.61	16.63 $\pm$ 3.11
DES 1.4	8	0.81 $\pm$ 0.14**	4.57 $\pm$ 0.92**
DES 5.6	6	1.03 $\pm$ 0.25**	4.00 $\pm$ 0.58**
DMSO-PBS	7	2.77 $\pm$ 0.18	16.95 $\pm$ 4.02
Estradiol-17 β	8	3.41 $\pm$ 0.14 ^{NS}	18.98 $\pm$ 3.33 ^{NS}

^aMale NMRI mice immunized ip with 0.3 ml 10% (v/v) SRBC 5 days before assay.

^bTreatment with 1.4 or 5.6 mg/kg/day DES sc, 48 mg/kg/day estradiol-17 β ip or vehicle, olive oil, and 4% (v/v) dimethylsulfoxide in PBS, respectively, from 2 days before immunization and 3 days thereafter.

^cNumber of mice in each group.

**0.01 > P $\geq$ 0.001.

^{NS}Not significant.

TABLE IV. Effect of Estrogen on the Primary Antibody Response to LPS^a

Treatment ^b	N ^c	Anti-LPS PFC	
		Per 10 ⁶ spleen cells (Mean $\pm$ SEM $\times 10^{-2}$)	Per spleen (Mean $\pm$ SEM $\times 10^{-3}$)
Olive oil	8	5.50 $\pm$ 0.71	29.69 $\pm$ 6.14
DES 1.4	8	3.39 $\pm$ 0.62*	19.19 $\pm$ 6.14 ^{NS}
DES 5.6	7	2.34 $\pm$ 0.20**	6.33 $\pm$ 1.03**

^aMale NMRI mice immunized ip with 10 μ g LPS 5 days before assay.

^bTreatment with 1.4 or 5.6 mg/kg/day DES sc from 2 days before immunization and 3 days thereafter.

^cNumber of mice in each group.

*0.05 > P $\geq$ 0.01.

**0.01 > P $\geq$ 0.001.

^{NS}Not significant.

interaction between adherent cells and specific T-helper cells [18]. In earlier studies we found that EMP treatment eliminated T lymphocytes in a dose-dependent fashion preferentially depleting the Ly 1⁺ subset [12]. Thus a numeric reduction of Ly 1 cells could at least partly be responsible for the reduced antibody response to SRBC following EMP treatment. Experiments on delayed hypersensitivity response did not indicate the presence of suppressor cells or humoral suppressor factors in mice treated with EMP [11]. Possible suppressor factors were not investigated in the present study. Adoptive transfer of T cells from EMP-treated B6 mice to the nu/nu B6 mice revealed that EMP-exposed T cells have an impairment in their ability to support a primary antibody response. Taken together, the results from experiments concerning the effect of EMP treatment on antibody response to a T-cell-dependent antigen suggest that EMP inhibits the response through elimination of T cells as well as impairing the function of residual T-helper cells.

The primary antibody response to the B-cell mitogen LPS is polyclonal and essentially thymus independent [19]. In the present study we examined the effect of EMP treatment on generation of antibody-producing cells with specificity for LPS. EMP exposure apparently did not have any significant effect on the number of anti-LPS PFC per 10⁶ spleen cells, while there was a significant reduction of PFC per spleen. This result should be considered in relation to the preferential elimination of T cells from the spleen after exposure to EMP [12]. The spleen of EMP-treated mice is relatively enriched for B cells [12], and the true effect of EMP may only be revealed when anti-LPS PFC are expressed per spleen. Thus EMP treatment probably impairs the total spleen B-cell response to T-independent antigens.

The mechanism underlying the effects of EMP on antibody response is not clear and seems to be different from that of estradiol. Extremely high doses of estradiol-17 β did not show similar effects. There is no settled view concerning the action of sex hormones on the antibody response. However, there is some evidence that pharmacological concentrations of estradiol-17 β inhibit humoral immunity [20,21]. The discrepancy between these findings and the present ones may be caused by differences in the antigen or in the schedule of estradiol treatment. The nor-nitrogen mustard moiety of EMP has alkylating properties and may exert its effect by inhibition of DNA synthesis in proliferating lymphocytes [22]. On the other hand, there is some evidence that the antitumor activity of EMP may be achieved by the intact estramustine molecule [23].

The sex difference in immune reactivity indicated by a more vigorous antibody response in females compared to age-matched males may reflect an immunomodulatory influence of sex hormones [24,25]. Estrogens, both natural and synthetic, are recognized stimulants of the reticuloendothelial system [26], and among these DES is one of the most potent [27]. On the other hand, DES suppresses cellular immunity [5,11] and causes thymic involution and severe depletion of cortical lymphocytes [28].

The present results confirm the findings of Luster et al [5] that DES exposure of adult mice inhibits the primary antibody response to T-cell-dependent as well as T-cell-independent antigens. Neonatal exposure to DES in female mice caused permanent disturbances in the humoral immunity of adult females [29]. Further penetration into the underlying mechanism revealed a defective T-helper cell function based on reduced proportion of T cells expressing the Ly 1 phenotype. The present results correspond well to these findings [29] and to our earlier reports showing selective depletion of Ly 1 cells from spleen after DES treatment of adult mice [12]. The conflicting data obtained in experiments with estradiol-17 β and DES treatment could indicate that beyond being a potent estrogen, DES or DES metabolites possess immunosuppressive properties independent of estrogen receptor-mediated mechanisms. In contrast to this assumption is the recent observation by Luster et al [30] that the estrogen antagonist clomiphene partially reversed DES-induced impairment of the immune response. However, the antiestrogenic properties of clomiphene have not been convincingly demonstrated in the mouse [31]. DES treatment also suppressed the primary antibody response and the proliferation of splenic lymphocytes to LPS, indicating an additional thymus-independent suppressive effect of DES treatment.

In summary, the present study shows that EMP as well as DES treatments of male mice are able to inhibit the primary antibody response both to thymus-dependent and thymus-independent antigen. A defective helper T-cell function is detected following EMP treatment.

ACKNOWLEDGMENTS

This investigation was supported by grants to project 1870-B83-O1XA from the Swedish Cancer Society and by AB LEO Research Foundation, Helsingborg, Sweden.

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Effects of diethylstilbestrol and estramustine phosphate (Estracyt[®]) on natural killer cell activity and tumor susceptibility in male mice

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Abstract

The effects of estramustine phosphate (EMP) and diethylstilbestrol (DES) on natural killer (NK) cell activity, tumor growth and artificial metastasis were investigated in male C57Bl/6 mice. Kinetic analysis and studies at the single cell level indicated that EMP did not influence the number of NK cells but interfered with their lytic activity thereby reducing the actual killer capacity. NK cells from EMP exposed animals responded normally to the interferon-inducer Poly I:C which restored NK activity to control levels. Spleen cells from DES treated animals had lytic activity comparable to that of control animals. However, more detailed analysis showed that DES reduced the number of lymphocytes able to recognize target cells, while the individual NK cell had an increased lytic activity and recycling capacity. Moreover, NK cells from DES treated animals were refractory to Poly I:C stimulation, suggesting that they were prestimulated *in vivo*. The perturbations of the NK cell system induced by both EMP and DES were reversible and normalization of NK activity reached within a week. The incidence of tumor takes after subcutaneous inoculation of the syngeneic Lewis Lung Carcinoma was increased in EMP as well as DES treated animals. Artificial lung metastasis produced by intravenous injection of the same tumor were increased in EMP but not in DES exposed animals.

Introduction

The highly regulated and organized network of the immune system and the rapid proliferation and differentiation of its cellular components make it susceptible to toxicological effects. Various forms of cancer therapy, including drugs, have important effects on host defense mechanisms with possible implications for defense against infections as well as spreading of tumor cells. Such effects may be specially prominent in the already immunocompromised cancer patient, and immunotoxicological effects of drugs should be a matter of increased concern in the treatment of tumors.

Patients with prostatic cancer have been shown to have impaired general as well as tumor associated immunity (1-3). The non-steroidal estrogen diethylstilbestrol (DES), one of the most widely used drugs for treatment of prostatic cancer, has been shown to further impair several aspects of the

immune response in cancer patients as well as experimental animals (4-6). During treatment, the proliferative response of the patients peripheral blood lymphocytes to certain T cell mitogens and the reactivity towards extracts of malignant prostatic tissue in the leukocyte adherence inhibition assay were reduced (7-8). Recently, DES was also found to profoundly impair Natural Killer (NK) cell activity (9). *In vitro* studies indicated that the NK cell was preferentially sensitive to DES among various lymphoid cells (10). Studies on estramustine phosphate (EMP), another commonly used drug for treatment of advanced or hormone resistant prostatic cancer are limited, but indicate that EMP has few immunotoxicological effects. In particular, no effect on human NK cell activity was found (9,11).

NK cells are presently believed to be of major importance in the control of metastasizing tumor cells and perhaps in immune surveillance of tumors (12-13). Moreover, NK cells have been reported to be of importance in the resistance against viral and some parasitic infections (14-15). The present paper is focusing on the effects of DES and EMP on NK cell activity in male mice and the possible relevance for defense against transplanted tumors and artificial metastasis.

Materials and methods

Animals

All mice used were inbred C57Bl/6 male mice. The strain was originally obtained from Anticimex, Sollentuna, Sweden and further propagated at our department. All mice entered the experiments at the age of 6-8 weeks and were strictly age matched.

Drug treatment

The animals were treated with diethylstilbestrol (DES, Sigma Chemicals Co, London) or estramustine phosphate (EMP, AB Leo, Helsingborg, Sweden) for 6 days and tested 24 hrs after the last drug injection. DES treated mice received 5.6 mg DES/kg body weight in 0.05 ml olive oil as a daily subcutaneous injection. EMP was given intraperitoneally twice a day at a daily dose of 30 or 100 mg EMP/kg body weight in saline. Control animals were given olive oil or saline only. Experimental and control animals were also studied 7 days after the last drug injection to establish the duration of the drug effects. Some animals were in addition given a single intraperitoneal injection of 100 μ g polyinosinic: cytidylic acid (Poly I:C, Sigma Chemicals) simultaneously with the last drug injection.

Natural killer cell assay

Effector cells

The animals were killed by cervical dislocation and a single cell suspension prepared from the spleen by pressing small spleen fragments through a stainless steel mesh. Red blood cells were eliminated by hypotonic shock treatment (16). In some experiments, effector cells were passed over a nylon-wool column before use (17).

Tumor cells

The Moloney murine leukemia virus-induced lymphoma YAC-1 was used as target for NK cells. The cell line was maintained as stationary suspension culture in RPMI 1640 culture medium (Flow Laboratories, Inc., Irvine, Scotland) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco, Biocult, Paisley, Scotland) 100 IU penicillin/ml and 100 μ g/ml streptomycin (complete medium). The cells were regularly screened for the presence of Mycoplasma and were always found to be negative. YAC-1 cells were labelled by incubation of 5×10^6 cells in 300 μ l RPMI 1640 medium with 150 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ (sp. act. 400-600 mCi/mg, Radiochemical Center, Amersham, England), for 45 min. at 37°C. The cells were washed 3 times in complete medium and adjusted to appropriate concentrations in complete medium.

Isotope release assay

NK cell activity was tested in a $^{51}\text{Chromium}$ release assay modified as described by Callewaert et al. (18) to determine kinetic parameters for NK activity analogous to enzyme-catalyzed reactions. In brief, the cytotoxic activity of a constant number of effector cells was tested against increasing concentrations of $^{51}\text{Chromium}$ labelled YAC-1 target cells. When the results are plotted as the rate of target cell lysis against the initial target cell concentration, the data fit a hyperbolic curve typical of Michaelis-Menten kinetics. For details, see Callewaert et al. (18,19). The assay was run for 4 hours with 10^6 effector cells and 2,4,6,8, and 10×10^5 target cells in 200 μ l complete medium per well in round-bottom microliter plates (Flow Laboratories, Inc. Irvine, Scotland). Kinetic parameters were calculated by distribution-free methods by the modification of a FORTRAN program obtained through the courtesy of Dr. D.M. Callewaert, Oakland University, Minnesota, and run on a UNIVAC 1100/80 computer.

Single cell assay

The assay was essentially as described by Grimm & Bonavida (20), with slight modifications. Spleen lymphocytes were prepared as described above and filtrated through nylon wool (17). Effector and target conjugates were formed by mixing equal numbers of effector cells and YAC-1 target cells (2×10^5 cells) in 200 μ l complete medium in conical 1 ml microflex tubes (Kontes Scientific Glassware, New Jersey, USA). The cells were incubated for 5 minutes at 30°C, spun at $500 \times g$ for 3 minutes and further incubated at 30°C for 10 minutes. The cells were resuspended by gentle pipetting 2 times with a pasteur pipette and added to 0.5 ml 0.5% agarose (grade A, Calbiochem, California, USA). The agarose was prepared in RPMI 1640 medium, melted in a boiling water bath and further kept in water bath at 45°C. Immediately before the addition of cells, the agarose was cooled to 39°C by exposure to room temperature. After admixture of the conjugated cells, small aliquotes of the agarose were dropped into plastic petri dishes precoated with agarose. The agarose was allowed to solidify for 1-2 minutes at room temperatur, covered with 2 ml complete medium and incubated at 37°C for 3 hours. Some plates were read immediately to determine the percentage of lymphocytes forming conjugates with target cells (by counting 400 lymphocytes) and at the end of incubation to determine the number of bound lymphocytes being cytotoxic (by counting 100 conjugates). Before reading, the plates were stained with trypan blue for 5 minutes and fixed with 0.5% formaldehyde in PBS.

Estimation of the percentage of active NK cells and mean recycling capacity (MRC)

The percentage of active NK cells was obtained from the single cell assay by multiplying the percentage of target binding cells with the percentage of conjugates with dead targets at the end of the assay. MRC was calculated by combining data from the isotope-release assay and the single cell assay. Thus, the $V_{\max}$ value was divided by the absolute number of killer cells, i.e. percentage of active NK cells multiplied with the number of effectors in the $V_{\max}$ (10^6). MRC is an estimation of the average number of target cells that an active NK cell can kill in 4 hours under optimal conditions.

Tumor transplantation

The Lewis Lung Carcinoma (LLC) was passed subcutaneously in syngeneic C57Bl/6 hosts by inoculation of a single cell suspension prepared by treatment of small tumor fragments with an enzyme mixture consisting of 0.1% collagenase, 0.1% hyaluronidase and 0.002% DNase (Sigma Chemicals) in PBS for 45 min. at 37°C. After 3 washes with complete medium the tumor cells were suspended in saline and either injected in normal hosts for further passage or 5×10^3 cells were injected subcutaneously (s.c.) in the flank of test animals for assay of tumor susceptibility 24 hrs after the last drug injection. Viability of LLC prepared in this way always exceeded 90% as determined by trypan blue dye exclusion. Inoculated animals were followed every day and the day of occurrence of a palpable tumor was recorded.

Artificial metastasis

Artificial metastasis were produced in animals treated with 5.6 mg/kg DES or 100mg/kg EMP daily for 6 days by intravenous (i.v.) injection of 10^5 LLC cells prepared as described above in 0.2 ml medium 24 hours after the last drug injection. Three weeks later, the animals were killed, the lungs removed and fixed in Bouins solution. Visible tumor colonies were counted under a dissection microscope.

Statistical analysis

Kinetic parameters for NK activity were compared using Students t-test. Before doing these comparisons it was checked that the error variances of the groups involved did not differ significantly. Tumor frequencies after subcutaneous transplantation were compared using the χ^2 test. The incidence of metastasis does not correspond to a normal distribution and was therefore analyzed with the Mann-Whitney U-test.

Results

Kinetic parameters of NK activity in male mice treated for 6 days with 30 or 100 mg/kg EMP and killed 24 hours after the last drug injection were analyzed in an isotope-release assay. The total lytic activity of spleen cells expressed as $V_{\max}$, was significantly reduced ($p < 0.01$) after treatment with 100 mg/kg EMP (Fig. 1). Exposure to 30 mg/kg EMP somewhat reduced $V_{\max}$, although the difference to control animals was not statistically significant. Exposure to the highest dose of EMP also reduced the K_M value of the NK cell-tumor interaction. Injection of the interferon-inducer Poly I:C 24 hours before the animals were killed strongly enhanced NK activity in control as well as EMP treated mice and $V_{\max}$ values of animals treated with 100 mg/kg EMP reached values comparable to that of vehicle exposed animals. K_M values were not affected by treatment with Poly I:C.

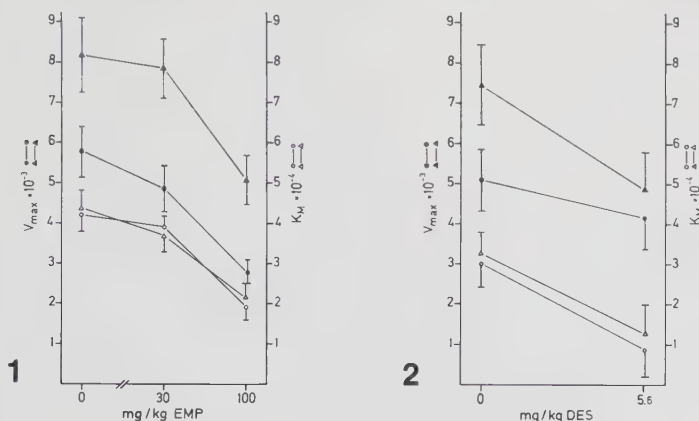


FIGURE 1: Kinetic parameters for NK activity against YAC-1 cells of spleen lymphocytes from male B6 mice given daily injections of 30 or 100 mg/kg body weight EMP for 6 days and assayed 24 hours after the last injection (circles). Some animals were in addition given a single i.p. injection of 100 µg Poly I:C simultaneously with the last injection (triangles). Each point represents the mean value $\pm$ SE of 5-7 animals assayed individually in 3 separate experiments.

FIGURE 2: Kinetic parameters for NK activity against YAC-1 cells of spleen lymphocytes from male B6 mice given daily injections of 5.6 mg/kg body weight DES for 6 days and assayed 24 hours after the last injection (circles). Some animals were in addition given a single i.p. injection of 100 µg Poly I:C simultaneously with the last injection (triangles). Each point represents the mean $\pm$ SE of 5-7 animals assayed individually in 2 separate experiments.

Activity of spleen NK cells of mice exposed to 5.6 mg/kg DES is shown in Figure 2. No significant alterations in $V_{\max}$ values were found while K_M were reduced as a consequence of DES treatment ($p < 0.01$). Additional treatment with Poly I:C resulted in a strong augmentation of $V_{\max}$ values in control animals but only in a slight enhancement of $V_{\max}$ in DES treated animals. K_M values were not altered by Poly I:C. The duration of the effects of EMP and DES was investigated in animals 7 days after the last drug injection and showed that this time interval was sufficient to completely restore deviations in NK activity (Table 1).

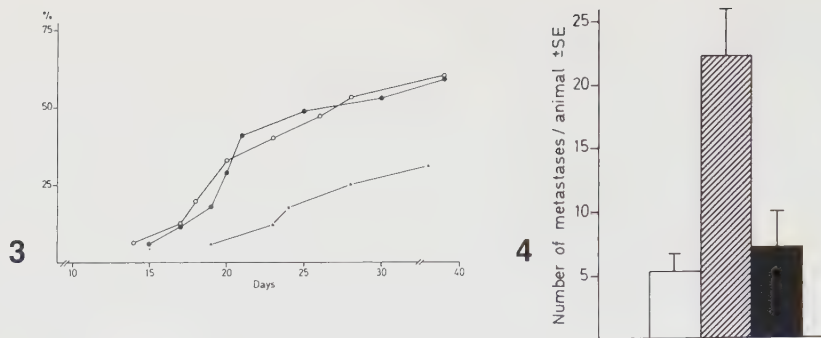


FIGURE 3: Cumulative incidence of palpable tumors after s.c. transplantation of 5×10^3 LLC cells to male B6 mice treated the preceding 6 days with vehicle (x), 100 mg/kg body weight EMP (o) or 5.6 mg/kg body weight DES (●). The number of animals in each group was 14-17.

FIGURE 4: The number of visible metastatic foci in the lungs 21 days after i.v. injection of 10^5 LLC cells of male B6 mice treated the preceding 6 days with vehicle (□), 100 mg/kg body weight EMP (▨), or 5.6 mg/kg body weight DES (■). The number of animals in each group was 10.

To get further insight into the mechanism of action of EMP and DES on mouse NK cells we applied a single cell assay in agarose with the ability to distinguish between two major steps in cell mediated cytotoxicity: binding of the effector cell to the target cell and the subsequent lysis of the latter. EMP inhibited the lytic activity of NK cells, but did not interfere with the number of spleen lymphoid cells actually binding a target cell (Table 2). In contrast, the number of lymphocytes able to bind the NK sensitive target YAC-1 was reduced to less than 50% of control values in DES treated animals. Moreover, the percentage of target recognizing lymphoid cells that went on to kill their target was increased. Thus, while the number of potential NK cells was decreased, the remaining cells were more efficient killers. NK cells may recycle, i.e. kill more than one target. The mean number of target cells lysed per NK effector was obtained by combining information of the lytic capacity and the number of active NK cells in the population (Table 3). These data indicated that DES increased the recycling capability of the individual NK cell.

TABLE 1

Normalization of NK activity in EMP or DES treated mice 7 days after cessation of treatment

Treatment	$V_{\max} \times 10^{-3}$	$K_M \times 10^{-4}$
Vehicle	4.8 ± 0.7	3.4 ± 0.4
EMP (100 mg/kg)	4.5 ± 0.5	3.6 ± 0.4
DES (5.6 mg/kg)	5.5 ± 0.7	3.1 ± 0.6

Male B6 mice were treated as indicated for 6 days and tested for NK activity against YAC-1 cells 7 days after the last injection. Each value represents the mean $\pm$ SE of 5-6 animals assayed individually in single experiment.

TABLE 2

Effect of treatment with EMP or DES on binding and lysis of YAC-1 tumor cells at the single cell level

	% target binding	% lysis of conjugated cells
Vehicle	9.7	26.0
	12.2	22.3
EMP (100 mg/kg)	7.1	18.5
	8.3	17.0
DES (5.6 mg/kg)	5.2	29.5
	4.4	32.7

Male B6 mice were treated as indicated for 6 days and tested for NK activity in single cell assay 24 hours after the last injection. Results from 2 separate experiments with pooled spleen cells from 2 animals per group are shown.

TABLE 3

Effect of treatment with EMP or DES on NK cell Mean Recycling Capacity (MRC)

Treatment	MRC
Vehicle	2.2
EMP (100 mg/kg)	2.0
DES (5.6 mg/kg)	2.8

MRC calculated from data presented in Fig. 1 & 2 and Table 2 as described in Materials and Methods section.

The syngeneic LLC to be used for assay of tumor susceptibility was found to be sensitive to NK mediated cytotoxicity, although somewhat less susceptible than the standard NK target YAC-1 (Table 4). S.c. inoculation of EMP or DES treated animals with a low number of LLC cells resulted in an earlier appearance and increased incidence of palpable tumors of treated compared with control animals (Fig. 3). Thirty % of control animals developed tumors at the end of the observation time while both EMP and DES treated animal groups had about 60% tumor bearing animals. Moreover, the number of metastatic foci in the lungs after i.v. injection of LLC cells was significantly higher in EMP exposed than in control animals ($p < 0.01$) (Fig. 4) DES treatment had no effect on the number of LLC establishing metastasis in the lungs.

TABLE 4

Sensitivity to NK mediated lysis of enzymatically prepared Lewis Lung Carcinoma (LLC) cells.

Target: effector ratio	% cytotoxicity	
	YAC-1	LLC
100:1	31.4	22.0
	27.5	18.7
50:1	20.6	14.5
	14.7	11.2
25:1	10.5	8.3
	10.1	7.2

Results from 2 separate experiments are shown.

Discussion

The present paper demonstrates that both DES and EMP have significant, although qualitatively different, effects on mouse NK cells after *in vivo* exposure. Only the highest dose of EMP tested, 100 mg/kg body weight, was found to be inhibitory. This dose of EMP has earlier been found to have preferential effects on mouse T lymphocyte associated functions evidenced as impairment of proliferative response to T cell mitogens, reduced number of antibody producing cells to T-dependent antigens, a reduced delayed hypersensitivity response as well as cytotoxic effects on T lymphocytes in lymph nodes and spleen (11). In contrast to the effect on mouse NK cells, NK activity in patients with prostatic cancer was unaltered during treatment with EMP (9). Moreover, human NK cells are resistant to EMP concentrations as high as 10^{-4} M *in vitro* (Kalland, in preparation). The discrepancies between the mouse and human system may be dependent on pharmacokinetic differences but intrinsic differences between mouse and human NK cells in the susceptibility to EMP should not be excluded. Inhibition of proliferative response to T cell mitogens *in vitro* is observed at 5×10^{-5} M EMP with human lymphocytes and at 10^{-5} M EMP with mouse lymphocytes (21,22). The effect of EMP was transient and full NK activity was reached within a week after cessation of treatment. NK cells belong to a rapidly renewing cell type in bone marrow and recovery from inhibitory influences is typically rapid with an intact bone marrow function (23).

More detailed information on the mechanism of effect of EMP on NK cells was obtained by combination of information obtained by kinetic analysis and studies at the single cell level of NK cell-tumor cell interactions. EMP did not significantly reduce the number of spleen lymphocytes binding YAC-1 target cells but affected their lytic capability, thereby reducing the number of active NK cells. It has been claimed that the K_M value obtained by Michaelis-Menten kinetical analysis of NK cell-target interaction reflects the percentage of active NK cells (19). In fact, in the present study a parallel decrease in the percentage of active NK cells determined by a single cell assay in agarose and the K_M value was obtained both after treatment with EMP and DES.

Interferon is a well known regulator of NK activity and interferon effects on NK cells *in vivo* include differentiation of pre-NK cells to lytically active NK cells, increase in the lytic activity of the individual NK cell and increase in their

recycling capacity (24-26). Injection of EMP treated animals with the potent interferon-inducer Poly I:C markedly increased NK activity and restored the $V_{\max}$ value to normal levels. This might indicate that EMP acts on mature cells leaving pre-NK cells with the capability to respond to interferon intact. Alternatively; stimulation of the lytic apparatus may be able to overcome the inhibition of lytic activity imposed by EMP, or stimulation of a subpopulation of NK cells resistant to EMP may be sufficient to restore a normal total lytic activity. The finding that Poly I:C treatment did not increase K_M value would favour the latter two interpretations.

Evidence has been presented that interleukin 2 (IL-2) stimulates NK cells and may be necessary to maintain the NK cell system *in vivo* (27,28). IL-2 has been shown to normalize the defect NK activity in the beige mouse mutant, known to have normal target binding NK cells but a defect in their lytic activity (29). Recently, we have shown that EMP inhibited lectin stimulated production or release of IL-2 from human lymphocytes *in vitro* (11). Whether this finding has any relevance for the observed inhibition of the lytic ability of mouse NK cells after *in vivo* exposure to EMP remains to be established, although notably, EMP in the mouse preferentially affects $Ly\ 1^+$ cells known to be IL-2 producers (22,30).

The total lytic activity of spleen lymphocytes against YAC-1 cells was not significantly altered by treatment with 5.6 mg/kg DES for one week. This exposure regimen has earlier been found to perturb the immune system by reduction of the lymphoproliferative response to T and B cell mitogens, to reduce the effector phase of the delayed hypersensitivity response, to reduce the percentage of T lymphocytes in the spleen and to affect hematopoiesis resulting in bone marrow hypocellularity and a decreased number of CFUs (4,6). The profound reduction in K_M values observed after DES treatment with normal $V_{\max}$ values indicate more subtle alterations in NK activity and incited more detailed studies on NK cells in these animals. Studies at the single cell level revealed that the actual number of target binding cells was clearly reduced. Since the lytic activity of the spleen cell population was normal, this would indicate that the killer efficiency of the individual NK cell was increased. This assumption was confirmed both by study of the percentage of target binding cells that actually killed their target, and by estimating the number of target cells lysed by an individual NK cell. The percentage of lymphoid-tumor cell conjugates with a dead target as well as the estimated mean recycling capacity of the individual NK cell was increased in DES treated animals. Injection with Poly I:C only slightly increased the lytic capacity of DES treated animals while strongly enhancing NK activity in control animals. Taken together, these data suggest that the NK cell system in DES treated animals is prestimulated with interferon *in vivo*. Since the actual number of NK cells was reduced, this effect may function as a compensatory mechanism. Macrophages regulate NK activity in positive and negative ways, the former via production of interferon (31). DES is well known as one of the most potent stimulators of macrophage phagocytic activity and induces increased production of plasminogen activator and increased ability to inhibit murine leukemia cell growth (6). A possible explanation of the observed dualistic effect of DES on NK cells would be that DES by inducing hypocellularity in the bone marrow also reduce the number of NK cells, but that stimulation of macrophage interferon synthesis stimulates those remaining.

Although NK cells have been implicated in the resistance against viral and certain parasite infections, the major interest in NK cells has been related to their possible relevance for the defense against tumors (13). To evaluate their

eventual role of the disturbances in immune regulation induced by EMP and DES, the resistance to tumor growth after subcutaneous or intravenous inoculation was studied. Exposure to both EMP and DES significantly increased the incidence of tumor take after subcutaneous transplantation of the syngeneic LLC tumor.

Increased tumor susceptibility after adult or neonatal exposure to DES have been reported earlier (32,33). In contrast, DES did not influence the frequency of metastatic foci in the lungs after i.v. injection. Artificial tumor metastasis have been found to be strongly dependent on the immune competence of the host and in particular on the level of NK activity (34-36). The lack of effect of DES on lung metastasis may therefore indicate that although the number of NK cells is reduced, the maintenance of a normal lytic capacity due to increased recycling capacity and killer ability is sufficient to prevent an increase in lung tumor growth. The discrepancy between the effect on subcutaneous tumor growth and artificial pulmonary metastasis may indicate that NK cells active at different compartments show different sensitivity towards DES or that tumor growth at these sites may be controlled by partly different defense mechanisms. Recently, it was reported that extension of the exposure time to DES to 2 weeks increased the host defense against B16-F10 cells after i.v. injection (37). This cell line has been shown to be susceptible to macrophage mediated cytotoxicity and it was suggested that prolonged treatment with DES resulted in activation of cytotoxic macrophages. EMP increased the susceptibility to s.c. transplanted as well as i.v. injected tumor cells and these findings further support the notion that the susceptibility to blood-borne pulmonary metastasis is related to host NK level. It should be pointed out, however, that the immunotoxic effects of neither EMP nor DES are specific for the NK cell system (4,11).

In summary, the present paper demonstrates that EMP and DES perturb NK cell activity by qualitatively different mechanisms and that exposure to these drugs have significant consequences for the defense against transplanted tumors. The relevance of these findings for the treatment of patients with prostatic cancer with EMP or DES is purely speculative. It may be concluded, however, that both drugs have immunotoxic properties and possible effects on the human immune system and host defense mechanisms should be further studied.

Acknowledgements

This investigation was supported by grants to projects 1870-B83-01XA from the Swedish Cancer Society, the Medical Faculty, University of Lund and AB LEO Research Foundation, Helsingborg, Sweden.

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In Vitro and In Vivo Effects of Diethylstilbestrol and Estramustine Phosphate (Estracyt®) on the Mitogen Responsiveness of Human Peripheral Blood Lymphocytes

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The responsiveness to diethylstilbestrol (DES) and estramustinephosphate (EMP) of human peripheral blood lymphocytes to T-cell mitogens has been investigated in vitro and in vivo. EMP demonstrated potent inhibition of both Con A- and PHA-induced lymphocyte proliferation in vitro, while it had no detectable effects when given to patients with cancer of the prostate. DES reduced the response to Con A in vitro, but had only marginal effects on PHA-induced mitogen response. In contrast, the response to Con A was unaltered, while the response to PHA was significantly diminished after DES therapy in patients with prostatic cancer. This effect, however, was only seen when high doses of DES not included in conventional regimen were given. The proliferative response to T-cell mitogens in patients with prostatic cancer was not affected by serum source in the assay, indicating the absence of humoral factors able to inhibit mitogen response in these patients.

Key words: diethylstilbestrol, estramustine phosphate, human lymphocytes, mitogen response, prostatic cancer

INTRODUCTION

Endocrine manipulation has become a generally accepted mode of therapy for advanced adenocarcinoma of the prostate [1]. The nonsteroidal estrogen diethylstilbestrol (DES) is probably the most widely used drug in the treatment of prostatic carcinoma because of the well-documented palliative effect and low cost. The estrogen-resistant tumors have been a challenge to the urologist, and in recent years, encouraging results in the treatment of estrogen-resistant prostatic carcinoma with the steroidal alkylating agent estramustine phosphate (EMP, Estracyt®) have been reported [2-5].

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The adverse effects of estrogenic therapy on the cardiovascular system are well known, but information on the effects of the immune system are generally lacking. An exception is the work of Ablin and associates who have shown that DES impaired general as well as tumor-specific immunity in patients with prostatic carcinoma [6-8]. Several investigators have reported that patients with advanced cancer of the prostate have disturbances in their immune response as judged by lymphocyte proliferation in response to mitogens and delayed hypersensitivity response to recall antigens [8-14]. Based on studies of mitogen response and lymphocyte reactivity to tumor antigens in patients treated with DES [6-8], concern has been expressed whether estrogens may add further to the impairment of the immunocompetence in these patients with possible consequences for tumor growth and dissemination, as well as for the defense against infections [15-17].

This study was undertaken to delineate the effect of DES and EMP on the mitogen-stimulated proliferation of peripheral blood lymphocytes from patients receiving treatment for prostatic carcinoma with either DES or EMP and to compare these effects with the inhibitory activity of the drugs *in vitro*.

MATERIALS AND METHODS

Twenty patients with a histologically or cytologically confirmed diagnosis of adenocarcinoma of the prostate, ranging in age from 61 to 83 years of age, and six healthy young men were included in this study.

The grade of malignancy of the tumors and clinical stage of the patients with prostatic cancer were evaluated as previously described [14]. Patients with stage I and II, well-differentiated or moderately differentiated carcinoma did not receive any treatment and are not included in this study. Seven patients with poorly differentiated carcinoma received 840 mg of EMP daily in three oral doses irrespective of clinical stage. Eight patients with stage III and IV, well or moderately differentiated carcinoma of the prostate received conventional estrogenic therapy according to our protocol [14]. This regimen consisted of oral treatment with 10 mg of DES daily for the first two weeks, thereafter 2 mg of DES daily. Together with DES therapy, the patients on conventional regimen received 80 mg (IM) of the long-acting estrogen polyestradiol phosphate (PEP, Estradurin®) on the first day of treatment and later every fourth week. Five patients with metastatic carcinoma of the prostate and severe pain from metastasis were selected for high doses of DES (Honvan®) therapy, administered intravenously as primary treatment and received 1,000 mg of DES IV daily for seven days. Mitogen response of the patients lymphocytes were tested immediately before and after four weeks of treatment. Patients given the high doses of DES were tested after a treatment period of one week.

Separation of Mononuclear Cells

Peripheral blood was obtained by venepuncture. Twenty to forty milliliters of fresh whole blood were collected into sterile tubes containing 100 IU of heparin (Novo Industri A/S, Copenhagen, Denmark; 5,000 IU/ml free of preservative) per ml blood and diluted 1:1 in physiologic phosphate-buffered saline (PBS, pH 7.4). Forty milliliters of diluted blood were layered onto 7.5 ml of a sodium metrizoate-Ficoll gradient solution (Lymphoprep®, Nyegaard & Co, Oslo, Norway) in 50-ml conical plastic tubes.

Mononuclear cells (peripheral blood lymphocytes, PBL) were separated accord-

ing to Bøyum [18]. Briefly, the gradients were centrifuged at 800g for 20 minutes at room temperature (22–24°C), and the mononuclear cells harvested from the interface between the blood sample and the gradient solution.

The mononuclear cell yield was 70% and the viability was above 90% as determined by the trypan blue dye exclusion test. The mononuclear cell suspension was washed twice in PBS, once in culture medium (see below) and the cell number adjusted to 2×10^6 cells/ml.

Mitogen Stimulation Test

Two million cells in 0.2 ml of medium were cultured per well in round-bottom microtiter plates (Grenier Original, Cambridge, Mass, USA) with or without mitogens and the drugs to be tested (see results).

The culture medium consisted of RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Gibco Bio-Cult, Peysley, Scotland) supplemented with 10% heat-inactivated human AB serum (pooled serum from AB RH-positive donors) or autologous serum.

Penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added to the medium. Triplicate cultures were run without mitogen and with various concentrations of the T-cell mitogens concanavalin A (Con A, Sigma Chemicals Ltd, St. Louis, Mass, USA; highly purified grade IV) and phytohaemagglutinin (PHA, Miles Laboratories, Revohot, Israel).

After culture for 48 hours, 0.5 µCi methyl-³H-thymidine (³H-dThd sp act 5 Ci/mM, the Radiochemical Center, Amersham, England) was added to each culture. The cells were harvested onto glass-fiber filters 24 hours later with a Skatron Cell Culture Harvester (Skatron A/S, Lierbyen, Norway). The filters were dried, put into counting vials containing 3 ml of scintillation fluid [19], and radioactivity measured in a Packard Tri-Carb liquid scintillator. The counting efficiency was 40% as determined by internal standard. To define which dose of the mitogen would give the optimal stimulation, cultures containing various amount of Con A or PHA were evaluated in initial experiments. From these experiments a concentration of 50 µg/ml of Con A and 5 µl PHA per well was chosen in the testing of the in vitro effects of DES or EMP on lymphocyte proliferation.

RESULTS

In Vitro Effects of DES and EMP on Lymphocyte Proliferation

The response of Con A-stimulated PBL from healthy young men and from patients with various stages of prostatic carcinoma before treatment showed the same trend, eg, a concentration-dependent inhibition of lymphocytic blastogenesis (Fig. 1 and 2a). The only exception of this general trend observed was that DES at a low concentration (0.12 mM) stimulated the mitogen response of healthy young men to some degree, while PBL from patients with prostatic carcinomas was inhibited at the same concentration of DES.

EMP proved to be a more potent inhibitor of lymphocytic blastogenesis than DES on an equimolar basis (Fig. 1 and 2). PHA-stimulated lymphocyte proliferation was only examined in patients with carcinoma of the prostate. As can be seen from Figure 2b, EMP strongly inhibited PHA-stimulated proliferation at concentrations above 0.12 mM, while DES at the same concentrations had no inhibitory effects on

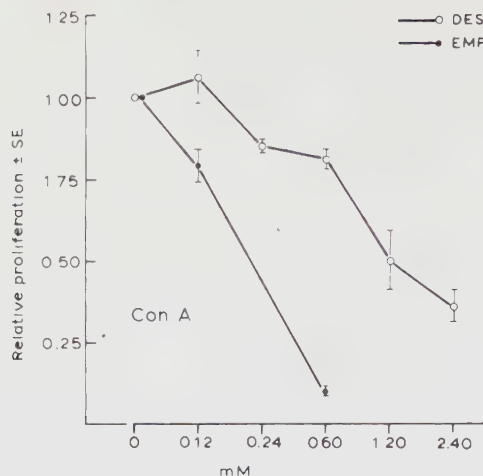


Fig. 1. Relative Con A-stimulated proliferation of PBL from six healthy males in the presence of various concentrations of DES or EMP.

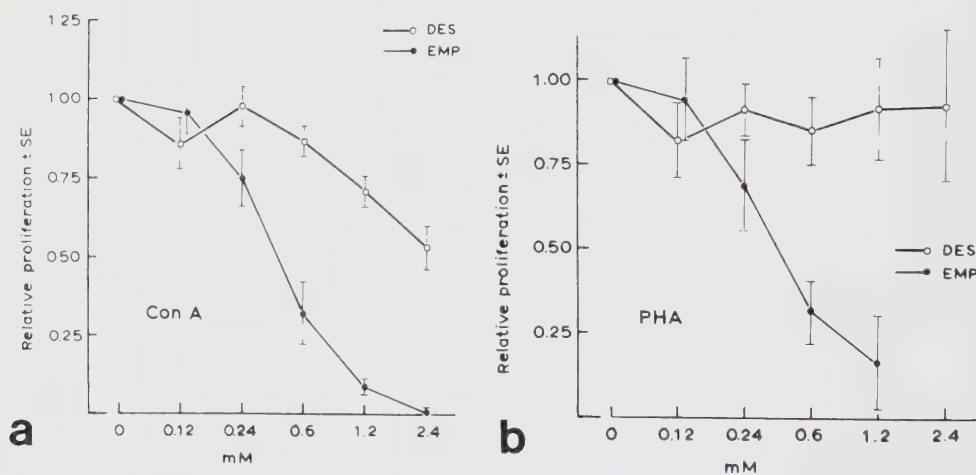


Fig. 2. a: Relative Con A-stimulated proliferation of PBL from 18 patients with prostatic cancer in the presence of various concentrations of DES or EMP. b: Relative PHA-stimulated proliferation of PBL from 9 patients with prostatic cancer in the presence of various concentrations of DES or EMP.

PHA induced proliferation. The in vitro effects of various concentrations of EMP and DES were also tested in mitogen-stimulated cultures of PBL from patients receiving either EMP or DES after four weeks of therapy. Neither Con A (Fig. 3 and 4) nor PHA (data not shown) response was significantly altered compared with the responses prior to therapy. The effect of DES in vitro on mitogen response of PBL before and after treating the patients with high dose DES was the same (Fig. 5).

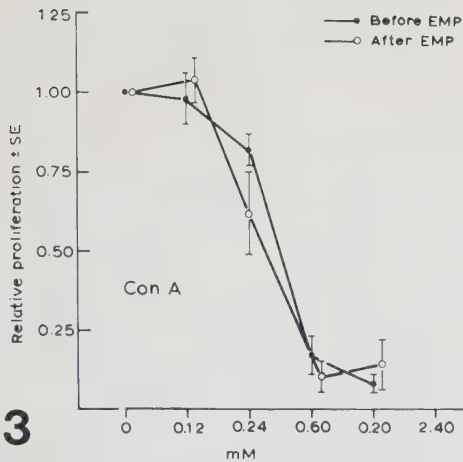


Fig. 3. Relative Con A-stimulated proliferation of PBL from eight patients in the presence of various concentrations of EMP before and after four weeks of EMP therapy.

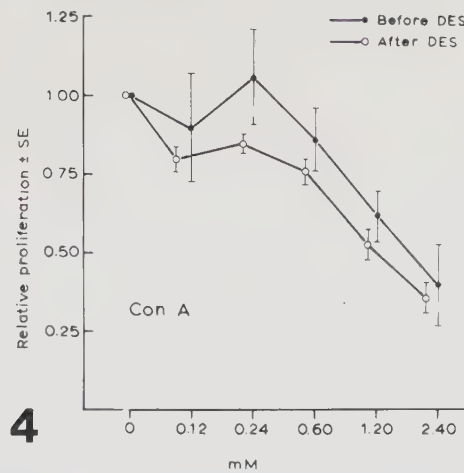


Fig. 4. Relative Con A-stimulated proliferation of PBL from seven patients in the presence of various concentrations of DES before and after four weeks of DES therapy.

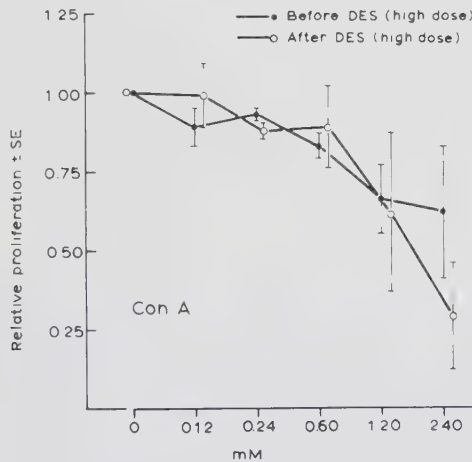


Fig. 5. Relative Con A-stimulated proliferation of PBL from three patients in the presence of various concentrations of DES before and after one week of high-dose DES therapy.

In Vivo Effects of DES and EMP on Lymphocyte Proliferation

The proliferative response to Con A of PBL from patients receiving EMP therapy was not significantly altered compared with the response before treatment (Fig. 6a, Table I). PBL from patients cultured in medium containing AB serum showed a trend consisting of inhibition of Con A-stimulated proliferation after EMP treat-

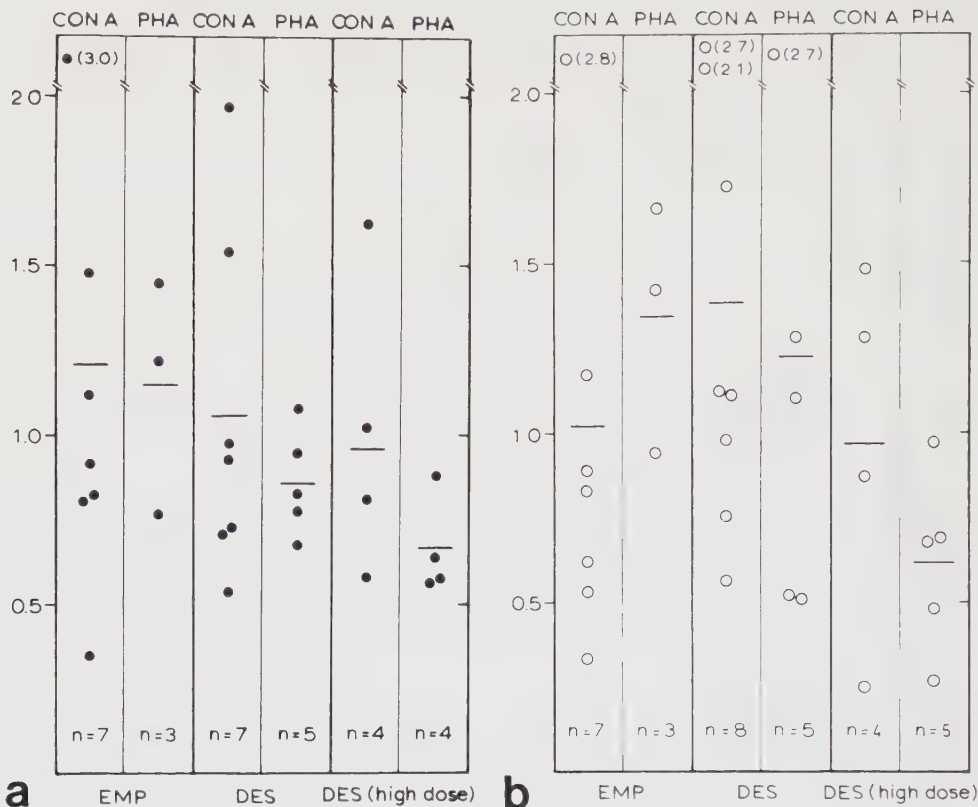


Fig. 6. a and b: Proliferation of PBL from patients with prostatic cancer stimulated with Con A or PHA in culture containing autologous (○) or homologous (●) serum after treatment with EMP, DES, or high-dose DES. The values were expressed as after-therapy related to pretherapy values. No change in proliferation during therapy would thus give a value of 1.0.

ment. Only three patients treated with EMP were examined for PHA responsiveness of their PBL before and after treatment; and no trend or significant alterations were observed (Table II). Figures 6a and 6b show the distributions of the values of the relative Con A- and PHA-stimulated proliferation of PBL from each patient before and after EMP treatment. PBL from one patient showed a substantial augmentation of Con A response after EMP therapy, while lymphocytes from the majority of patients responded less well to Con A after than before EMP therapy.

The mitogen response induced either by Con or PHA was not significantly altered after DES therapy for four weeks (Table I and II). The values of relative Con A-stimulated proliferation of PBL from each patient prior to and after DES treatment were widely spread independent of serum source used in the medium (Fig. 6a,b). The mean value of relative proliferation of PBL from DES-treated patients cultured in AB serum medium was 1.4, indicating a tendency towards an enhancing effect on Con A responsiveness. Lymphocytes from DES-treated patient showed some degree of inhibition of PHA-stimulated proliferation compared with the proliferation of PBL before treatment (Table I and II).

TABLE I. Con A Response of Peripheral Blood Lymphocytes From Patients With Prostatic Cancer Before and After Various Treatment Modalities

Treatment	n ¹	Serum source	³ H-dThd incorporation (mean Δ cpm $\pm$ SE)	
			Pretherapy	Posttherapy
Estracyt	7	Autologous	43,251 $\pm$ 7,188	43,195 $\pm$ 8,149
Estracyt	7	AB	45,122 $\pm$ 4,631	41,030 $\pm$ 9,994
Diethylstilbestrol Estradurin	7	Autologous	42,468 $\pm$ 10,769	39,637 $\pm$ 10,936
Diethylstilbestrol Estradurin	8	AB	39,989 $\pm$ 14,138	47,617 $\pm$ 10,884
Honvan	4	Autologous	40,184 $\pm$ 8,003	35,230 $\pm$ 4,785
Honvan	4	AB	28,691 $\pm$ 7,453	33,496 $\pm$ 12,102

¹n = number of patients in each group of treatment.

TABLE II. PHA Response of Peripheral Blood Lymphocytes From Patients With Prostatic Cancer Before and After Various Treatment Modalities

Treatment	n ¹	Serum source	³ H-dThd incorporation (mean Δ cpm $\pm$ SE)	
			Pretherapy	Posttherapy
Estracyt	3	Autologous	36,684 $\pm$ 14,744	36,394 $\pm$ 7,263
Estracyt	3	AB	34,133 $\pm$ 12,877	40,544 $\pm$ 8,028
Diethylstilbestrol Estradurin	5	Autologous	61,920 $\pm$ 9,664	54,550 $\pm$ 12,240
Diethylstilbestrol Estradurin	5	AB	54,747 $\pm$ 13,611	61,168 $\pm$ 20,058
Honvan	4	Autologous	66,447 $\pm$ 12,182	46,510 $\pm$ 12,732
Honvan	5	AB	62,192 $\pm$ 11,962	40,324 $\pm$ 9,666*

¹n = number of patients in each group of treatment.

*P < .05

High-dose DES administered intravenously for a week consistently inhibited PHA-stimulated lymphocyte proliferation, and the observed inhibition of PHA response after high-dose DES therapy was statistically significant ($P < 0.05$) (Table II). There was no difference in the PHA response according to whether the culture medium contained autologous or AB serum. Comparing in vitro sensitivity to DES and EMP before treatment with the actual alterations in response during therapy gave no indication that in vitro sensitivity to the drug could be used as a predictive test to foresee the effect in the individual patient in vivo.

DISCUSSION

Mitogens are polyclonal activators of lymphoid cells and have been widely used for the characterization of subpopulations of lymphocytes and as a measure of general immunocompetence [20].

The present study demonstrates that DES and EMP in vitro suppress Con A- and PHA-stimulated proliferation of lymphocytes from healthy male subjects and patients with carcinoma of the prostate. Estrogens have in several studies been reported to inhibit various aspects of the immune response in vitro, including proliferative response

to T-cell mitogens, mixed lymphocyte cultures, and antigen-specific leukocyte adherence inhibition [6,7,15–17]. The mechanism behind the immunosuppressive effects of estrogens is not clear. Estrogen receptors have not been detected in resting or PHA-stimulated lymphocytes, and DES does not cross-react with glucocorticoid receptors mediating the immunosuppressive action of glucocorticoids [21]. The observation that higher than 10^{-5} M DES is necessary to induce inhibition of mitogen response adds further to the argument against a receptor-mediated mechanism. Alterations in calcium flux through the lymphocyte membrane have been suggested to be an early step in mitogen-induced proliferation, and estrogens may prevent the entry of calcium into lymphocytes [22]. Estrogens also inhibit glucose and nucleoside transport [23–24] in various cells, and effects on precursor pools should not be excluded. Another possibility is that DES in similarity with steroidal estrogens may incorporate into membrane lipids modifying membrane properties [25].

It was not possible to abrogate completely the mitogen response with DES. DES may act preferentially on some of various signals delivered by lectins to induce lymphocyte proliferation. On the other hand, various subpopulations of T lymphocytes may have different sensitivity towards DES. For example, DES treatment of neonatal mice preferentially affects T helper cells [26], and in the present study, high-dose DES therapy inhibited the PHA response leaving the Con A response unaltered. In fact, T helper cells (T cells with receptor for IgM) are the major responders to PHA [27].

The relation between DES concentrations able to inhibit mitogen response in vitro and the actual serum levels reached during treatment is of considerable interest. Some estimates of serum DES levels after oral administration are available but differ considerably among various studies as well as among individuals [28–30]. Abraham and associates have reported peak serum levels of DES in the range of 10^{-8} M after the ingestion of a 10-mg dose [28], while Guinan et al estimates a serum level of 10^{-4} after an equivalent dose [29]. Comparisons of the in vitro and in vivo data from the present paper indicate that conventional oral doses of DES are not sufficient to perturb mitogen responsiveness, while high intravenous doses recommended for selected cases with extensive disease may affect some subpopulations of T lymphocytes. However, various aspects of the immune response may show different sensitivity towards DES. The conventional oral DES regimen has been shown to reduce the activity of natural killer cells [31], while the exact treatment modality was not given in a series of papers demonstrating effects of DES treatment on mitogen response and leukocyte adherence inhibition [6–8].

Studies on the effects of EMP on the immune system are severely lacking. Studies in patients with prostatic carcinoma [2–5] have revealed that EMP has low bone marrow toxicity, and Küss et al [32] found no significant changes in either cutaneous response to recall antigens or in serum immunoglobulin level after three months of therapy with EMP. Similarly, a recent study from our laboratory could not detect any effects of EMP treatment on natural killer cell activity, known to be strongly bone marrow dependent [31,33]. The present study demonstrates that EMP in vitro is completely inhibitory to both Con A- and PHA-induced lymphocyte proliferation in millimolar concentrations. The alkylating moiety of EMP is released after metabolism in the cultures (unpublished observation) thus inhibiting DNA synthesis and cellular proliferation. This assumption is supported by findings in male mice where EMP exerted a strong inhibitory influence in the sensitization phase of the delayed hypersensitivity response to oxazolone, which is known to be dependent on cellular proliferation but

was without effect in the effector phase [34]. Cyclophosphamide, another alkylating agent, also inhibited the response to Con A and PHA in both animals and man [36]. Moreover, estradiol at equivalent concentrations had no effect on mitogen response [35]. EMP has been shown to preferentially eliminate T lymphocytes from the spleen of male mice, and thus, a simple reduction in the number of viable T lymphocytes might explain the reduced response to T-cell mitogens [35].

In contrast to the results from animal studies as well as the *in vitro* data, EMP does not seem to affect the mitogen response in patients treated for cancer of the prostate. Differences with regard to drug metabolism and dose-response relations make the situation unclear.

Humoral blocking factors at least partly responsible for the diminished lymphoproliferative response to mitogens seen among patients with various forms of malignancy have been reported by several authors [for review see 37]. In the present study, the pretherapy and posttherapy response in autologous or pooled AB serum was compared. The pretherapy response to Con A and PHA was independent of serum source in the culture; thus, we were not able to detect humoral suppressor factors in patients with prostatic cancer of different stages. Controversy exists regarding the presence of humoral suppressor factors in patients with prostatic cancer [38–39].

In summary, the present study demonstrates that EMP is a potent inhibitor of mitogen-induced lymphocyte proliferation *in vitro*, while it had no detectable effects when given to patients with prostatic carcinoma.

DES reduced the response to Con A *in vitro* but had only marginal effects on PHA-induced mitogen response. In contrast, the response to Con A was unaltered, while the response to PHA was significantly diminished after DES therapy in patients with prostatic carcinoma. This effect, however, was only seen when high doses of DES not included in conventional regimen were given.

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EFFECT OF TREATMENT WITH DIETHYLSTILBESTROL-POLYESTRADIOL PHOSPHATE OR ESTRAMUSTINE PHOSPHATE (ESTRACYT) ON NATURAL KILLER CELL ACTIVITY IN PATIENTS WITH PROSTATIC CANCER¹

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ABSTRACT

We evaluated the effect of treatment of patients with prostatic cancer with estramustine phosphate or the combination diethylstilbestrol-polyestradiol phosphate on the natural killer cell activity in peripheral blood. Although estramustine phosphate did not affect natural killer cell activity, diethylstilbestrol-polyestradiol phosphate substantially reduced natural killing after a treatment period of 1 week. The activity was only slightly further lowered after 4 weeks of treatment. Possible clinical implications of the difference in susceptibility of natural killer cells to these agents are discussed.

Estrogenic therapy is generally accepted as a mode of treatment for advanced prostatic cancer, effective mainly through its androgen-depleting action (1). Although effects of estrogens on the immune system are well documented in experimental studies, possible implications for patients with prostatic cancer treated with estrogens have mostly been ignored (2, 3). An exception is the work of Ablin and associates who showed that the nonsteroidal estrogen diethylstilbestrol (DES) impairs general as well as tumor-specific immunity in patients with prostatic cancer (4, 5).

A recent advancement in tumor immunology was the discovery of lymphoid cells' ability to lyse a variety of tumor cells in vitro without prior sensitization (6, 7). The cells responsible for this spontaneous cytotoxicity were termed natural killer (NK) cells and are distinct from conventional T lymphocytes, B lymphocytes, or macrophages. The NK cell bears surface markers which indicate that it may be an early cell in the T cell lineage, but also seems to share characteristics with promonocytes (8, 9).

NK cells have been reported to play a role in the resistance to tumors in vivo (10-12). Recently, the mouse mutant *beige* has been shown to be severely deficient in NK function (13). The *beige* mouse shows increased growth rate, faster induction time, and increased metastatic capability of NK-sensitive tumors (14). This study deals with the effects on NK activity in patients treated for prostatic cancer with the steroidal alkylating agent estramustine phosphate or the combination of DES and the long-acting estrogen polyestradiol phosphate.

MATERIALS AND METHODS

Patients. Eleven patients with a confirmed diagnosis of prostatic adenocarcinoma were included in this study, ranging from 61 to 79 years of age.

Submitted for publication June 20, 1980; accepted September 29, 1980.

¹ This study was supported by grants from The Norwegian Cancer Society (Landsforeningen mot Kreft) and A/B LEO Research Foundation, Helsingborg, Sweden.

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The grade of malignancy and clinical stage of the prostatic cancer were evaluated as described (15). Patients with stage I and II, well or moderately differentiated carcinoma, received no treatment and are not included in this study. Patients with poorly differentiated carcinoma received primary treatment with a hormone-cytostatic complex, estramustine phosphate (EMP, Estracyt), irrespective of clinical stage (four patients). The treatment consisted of 840 mg of EMP daily in three oral doses. Patients with stage III and IV, well or moderately differentiated carcinoma, received conventional hormonal treatment with DES and polyestradiol phosphate (PEP, Estradurin) (five patients). The regimen consisted of oral treatment with 10 mg of DES daily for the first 2 weeks, and thereafter 2 mg of DES daily. PEP was given at the start of treatment (80 mg im) and later every 4 weeks.

Two patients with advanced prostatic cancer were selected for high dose DES treatment intravenously as primary treatment, and received 1000 mg of DES daily for 1 week. NK activity was determined immediately before the start of treatment as well as 1 and 4 weeks later.

Effector cells. Mononuclear cells from peripheral blood were separated on Lymphoprep (Nyegaard, Oslo, Norway) according to Böyum (16). The cells were washed three times in phosphate buffered saline (PBS, pH 7.4) and resuspended in Roswell Park Memorial Institute (RPMI) 1640 with 20 per cent human AB-serum to 10^7 cells per ml. In preliminary experiments, plastic adherent cells were found to exert only negligible cytotoxic activity in the present assay system, and filtration through nylon wool increased the cytotoxic activity. The per cent cytotoxicity was close to linear at effector-to-target ratios between 100 and 10.

Target cells. MOLT-4, a T-lymphoblast cell-line originally established from a patient with acute lymphoblast leukemia (17), was maintained as a stationary suspension culture in RPMI 1640 medium supplemented with 10 per cent fetal calf serum.

Cytotoxicity assay. Target cells were labeled by incubation of 5×10^6 cells in 0.5 ml of complete medium with $100 \mu\text{Ci}$ of $\text{Na}_2^{51}\text{CrO}_4$ (sp act 200 to 400 mc per mg, The Radiochemical Center, Amersham) for 45 min at 37 C. The cells were washed twice in 50 ml of phosphate buffered saline and resuspended in RPMI 1640 to 10^5 cells per ml. Target cells ($100 \mu\text{l}$) were admixed to effector cells ($100 \mu\text{l}$) in Greiner round bottom

microtiter plates to give an effector-to-target ratio of 100:1, and the mixture was incubated at 37 C in 5 per cent CO₂ for 5 hr. After centrifugation at 500 × *g* for 10 min, 100 μl of the supernatant were pipetted off and the radioactivity determined in a Searle 1185 Gamma spectrophotometer.

Spontaneous release was determined by incubation of labeled target cells in medium only, and varied from 10 to 20 per cent of total radioactivity. Total radioactivity was determined by counting samples of the labeled target cells. Variations between triplicates was below 5 per cent. Percent cytotoxicity was determined by:

$$\frac{\text{test counts/min} - \text{spontaneous counts/min}}{\text{total radioactivity}} \times 100.$$

The percentages of cytotoxicity before therapy ranged from 14 to 42 (mean 31). No correlation with grade or stage of the prostatic cancer could be noted in this small patient group. NK activity was expressed as a percentage of the pretherapy level for the individual patient, thus each patient served as his own control.

RESULTS

The cytotoxicity of NK cells from patients with prostatic cancer treated with hormones or a hormone-cytostatic complex before and during treatment is shown in Figure 1.

EMP apparently did not influence NK activity as the level 1 and 4 weeks after initiation of therapy was the same as the pretherapy level.

Hormonal treatment consisting of a combination of DES and PEP substantially reduced natural killing after a treatment period of 1 week, whereas the activity was only slightly further lowered 3 weeks later. In one patient a more pronounced decrease between 1 and 4 weeks occurred. The two patients on high dose DES treatment both showed a decreased response when the therapy was changed after 1 week of treatment. The

small number of patients included in this study did not allow an evaluation of a possible dose-response relationship of DES on NK activity. Thus the results indicate that DES-PEP and high dose DES treatment significantly reduced NK activity compared to pretherapy levels in contrast to treatment with EMP.

DISCUSSION

EMP has estrogenic properties (18), and estrogens have been reported to inhibit NK activity after continuous high-dose treatment in the mouse (19). Moreover, the alkylating agent cyclophosphamide depressed NK activity in the mouse in a dose-dependent manner (20). Species differences in pharmacokinetics and metabolism may contribute to the lack of effect of EMP (21). Bone marrow related side effects of EMP treatment are rare (18), and Küss et al. found no effects on immunity as judged by the cutaneous response to recall antigens and the total immunoglobulin level in 30 patients treated with EMP for prostatic cancer (22). Recent results from our laboratory, however, indicate that EMP may impair the proliferative response of peripheral blood lymphocytes to T cell mitogens in some individuals with prostatic cancer (Haukaas et al., unpublished observation).

Work by Ablin et al. has provided evidence that DES treatment of patients with prostatic cancer may have unwanted effects on the immune system (4, 5).

Concern has been expressed whether DES may further debilitate the patient by interference with immune competence (23). This report adds further information to the earlier observed effects of hormonal treatment on the immune system in patients with prostatic cancer, showing that DES in combination with PEP or alone reduced the NK activity in peripheral blood.

Macrophage related suppressor cells regulating NK activity have been described (24). In the mouse DES activates the reticuloendothelial system and can induce suppressor cells inhibiting lymphocyte proliferation (25, 26). Similar mechanisms may be operating in patients on DES. Exposure of neonatal female mice to DES induced persistent disturbances in several branches of the immune response including a decreased NK activity (27-29).

Implantation of mice with an estradiol pellet reduced the NK activity after 4 to 6 weeks, probably through an effect on bone marrow cells (19). Moreover, DES inhibited lymphocyte functions *in vitro* (30) and a direct effect of DES on NK cells or their precursors in the bone marrow should not be excluded.

Accumulating evidence indicates that the NK cell plays a key role in immunologic surveillance of neoplasia, and may also be of major importance in the control of metastatic spread of cancer cells (10-14). A role for NK cells in metastasis was initially suggested by the observation that transplanted tumors rarely metastasize in nude mice that have high levels of NK activity (6). Also there is a direct correlation between the level of NK activity in different mouse strains and the short-term survival of tumor cells labeled with ¹²⁵I-Iododeoxyuridine with a lung colony assay (31). An important role of NK cells in determining the metastatic seeding of tumor cells is suggested from experiments with the NK deficient mutant *beige* which have a marked increase of metastasis after tumor transplantation (14) and from the finding of an increased resistance to NK cell lysis of metastatic cells compared to cells of the local tumor growth (32).

Whether the difference in susceptibility of NK cells to DES-PEP and EMP has any biological significance remains to be established. However, EMP may compare favorably to DES, especially in the treatment of tumors with high metastatic potential, e.g., poorly differentiated tumors. This assumption is in accordance with the recommendation of Jönsson (33) of EMP as the primary treatment of high grade prostatic carcinomas.

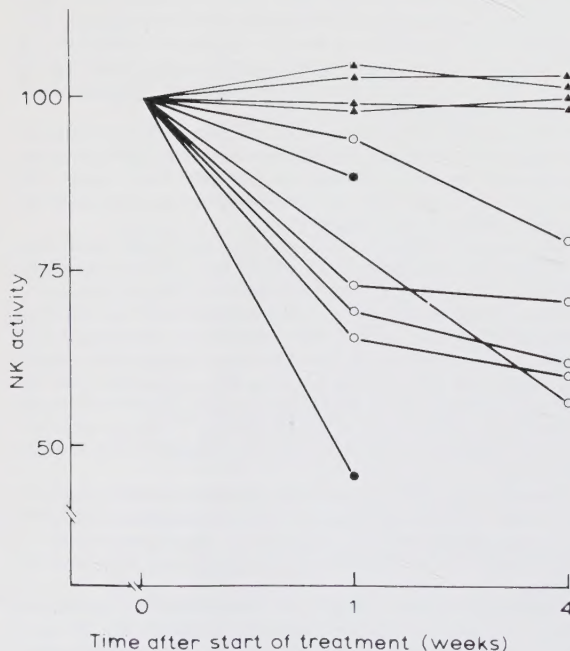


FIG. 1. Natural killer (NK) cell activity in peripheral blood of patients treated for prostatic cancer with EMP (▲), DES-PEP (○) or high dose DES (●). The activity after 1 and 4 weeks of treatment is related in percent to the pretherapy level.

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